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약학석사학위논문

Orally delivered DEVD peptide prodrug for metastatic pancreatic cancer in combination with Olaparib

전이성 췌장암에 대한 올라파립과 경구 DEVD 펩타이드 전구약물 병용요법

2021 년 1 월

서울대학교 대학원
분자의학 및 바이오제약학과

안상민

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Abstract

Orally delivered DEVD peptide prodrug for metastatic pancreatic cancer in combination with Olaparib

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Drug resistance is a major barrier in the treatment of anticancer drugs. The underlying cause of this drug resistance is tumor heterogeneity. In particular, intratumoral heterogeneity has been an obstacle to continuous anticancer treatment, showing resistance subgroup selectivity as drug treatment is repeated.

In order to avoid drug resistance due to tumor heterogeneity, this paper presents a DEVD peptide drug conjugate. DEVD prodrugs use an induced phenotype targeted therapy, which targets apoptosis and uses the fact that caspase-3 released in the apoptosis process specifically recognizes and cleaves DEVD and causes activation of prodrug. In order to apply this strategy to metastatic pancreatic cancer, we devised a combination of the targeted treatment, Olaparib.

In addition, a DEVD prodrug was orally administered to treat metastatic cancer based on oral metronomic maintenance therapy. The OPD3 complex was synthesized using DCK by taking advantage of the fact that the complex of bile acid-derived substances promotes oral absorption. Through this study, the pharmacokinetic parameters and absorption mechanism of OPD3 were identified, and

the tumor suppression ability was evaluated in a pancreatic cancer mouse xenograft model by combination therapy with olaparib. Because olaparib can be used specifically in the BRCA gene mutation patient group, the efficacy over BRCA mutated metastatic pancreatic cancer was evaluated. In addition, an attempt was made to establish a liver metastasis model, the most frequent metastatic organ of pancreatic cancer. Ultimately, we suggested the possibility of combination therapy of OPD3 and olaparib for BRCA gene mutant liver metastatic pancreatic cancer, which avoids drug resistance and has low systemic toxicity.

Keyword : Tumor heterogeneity, DEVD, Metastatic pancreatic cancer, Metronomic maintenance chemotherapy, Bile acid derivatives, Olaparib

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Chapter 1. Introduction

1.1 Conventional Cancer therapy

Cancer has been great threat to the mankind, thus efforts to conquer cancer has long history. From early nineteenth century, there was radical development in science. It allowed emergence of stable anaesthetic agents leading to improvement in surgical treatment of cancer. Also there was numeral findings of anti-cancer materials which kills rapid growing cancer cells. Additionally, the medical use of radiation expanded its extent to local treatment of cancer directly[1]. Over few decades, the use of surgical, chemical, radiation therapy maintained without any astonishing discovery, without only suggesting combination of existing therapies or anti-cancer drugs. Among the three mentioned therapy, chemotherapy is the only non-invasive pharmacotherapy. Chemotherapy targets fast proliferating characteristic, which is main difference of cancer cells from normal cells. Chemotherapy agents inhibit cell proliferation by various way; DNA alkylating, DNA gyrase inhibiton, tubulin inhibition.

Since cancer physiology advances, the hallmarks of cancer have been proved. Such cancer specific characteristics are sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death[2]. More than general characteristics, scientists found detailed features of cancer biology. These findings include proliferative signal receptors, apoptosis evading signal transducers. The next generation medicine after chemotherapy agents targets these cancer specific biologic targets. Such group of agents are called targeted therapy. Targeted therapy is composed of 2 types; Monoclonal antibodies and small molecules. Monoclonal antibodies targets specific protein or substance outside of the cancer cell. Also monoclonal antibodies are used to carry toxic chemo-drugs directly to the cancer. Angiogenesis inhibitor or

tyrosine kinase inhibitors are small molecule targeted therapy. These type of drugs block process related to the cancer cell proliferation.

1.2 Limitations of conventional cancer therapy

With the use of chemotherapy and targeted agents, there was significant limitations for each type of drugs. Chemotherapy has systemic toxicity and drug resistance problem. Targeted therapy, on the other hand, solved the systemic toxicity problem, but it still could not deal with drug resistance.

1.2.1 Systemic toxicity

Conventional Chemotherapy drugs have many systemic adverse reactions. This is due to the characteristic that chemotherapy agents target rapid growing cells. There are also normal rapid proliferating cells such as cells at hair, bone marrow, gastrointestinal tract, and reproductive organs. Side effects on such organs can lead to systemic toxicity of hair loss, myelosuppression, neutropenia, gastrointestinal distress, nausea, vomiting, infertility. These adverse events are main obstacle for patients to endure cancer treatment. Thus delivery system to minimize off-target drug action have been developed.

To overcome systemic toxicity from conventional chemotherapy, targeted drugs have been developed. Targeted drugs differ from chemotherapeutic drugs in that they have specific biologic target such as receptors or ligands which are related in the proliferation or apoptosis evading process. Therefore, targeted drugs can specifically be delivered to cancer tissue, avoiding drug action on normal cells. In that, the development of targeted drugs was great advance in cancer treatment history.

1.2.2 Drug resistance

Other than the toxicity, anti-cancer agents also had problem of resistance. Drug resistance in chemotherapy drugs are caused by increased drug inactivation or decreased drug activation, shift in drug target, drug efflux, and DNA damage repair[3].

First, anti-cancer drug can lose their efficacy by drug inactivation. For example, platinum-resistance can be induced by upregulation of methallothionein and glutathione, which plays role in inactivation of drugs. Also irinotecan resistance can be obtained via down regulation of UGT1A1 expression, which is crucial in activating irinotecan. Not only genetic resistance to certain drugs, epigenetic changes in cancer biology can give resistance to specific drugs.

Second, changes in drug target can cause drug resistance. Anti-cancer drug targets such as DNA gyrase or EGFR tyrosine kinase, cell proliferation signaling pathway proteins like MEK, Raf, Ras can efficiently modulated in the early drug treatment stage. However, as the treatment persist, cancer adjust to the environment and evade tumor suppression process by altering the main targets of the drug.

Moreover, drug efflux system can help cancer cell from affected by anti-cancer drugs. Many normal cells already have efflux system such as ATP-binding cassette (ABC) transporter to efflux toxins from accumulation. But in case of cancer cells, they express breast cancer resistance protein (BCRP), multidrug-resistance protein 1(MDR1), and multidrug resistance-associated protein 1(MRP1) to efflux anti-cancer drugs. These mechanism is specifically more critical to first generation chemotherapy agents.

Additionally, DNA repair system of cancer cell is important in responding to DNA damaging anti-cancer drugs. DNA damage caused by DNA damaging drugs can be reversed by DNA repair system of cancer cell. Many classical drugs damage DNA, so this mode of action was critical to some drugs. Thus, targeting drugs

that attacks DNA repair system has been developed recently.

1.3 Tumor heterogeneity

Variety of tumor clonal subpopulation is fundamental cause of resistance in both chemotherapeutic agents and targeted therapy. Cancer is product of continuous mutation in proto-oncogenes and tumor suppressor genes. Through such mutations, cancer develops heterogeneous subpopulations in single tumor tissue. This difference can be caused from diverse way; genetic, transcriptomic, epigenetic, and phenotypic alterations[4].

Briefly, there are 4 types of heterogeneity. Most common type is interpatient tumor heterogeneity. This type of heterogeneity stands for that no two patients have exact same tumor behavior, which results from the host factors such as tumor microenvironment and germline variants. Intratumor heterogeneity exhibits the distinct cellular populations with specific genetic, epigenetic, and phenotypic characteristics within cancers. In variety of tumors, including lung, breast, ovarian, pancreatic, kidney, intratumor heterogeneity has been recognized. In addition to the differentiation within specific tumors, tumors between metastatic lesions shows distinct cellular populations, which is called intermetastatic heterogeneity. Lastly, evolution within the metastatic lesions bring heterogeneity, which is known as intrametastatic heterogeneity. It is regarded as mechanism of acquiring drug resistance in metastatic cancer [5].

Over the 4 types of the tumor heterogeneity, intratumor heterogeneity is greatly associated with clonal evolution of tumor and its disease progression. Therefore, this article will concentrate on intratumor heterogeneity. Tumor heterogeneity already exist in the primary tumor because of genetic instability in proliferating cancer cells. Therefore, there might be dominant sub population which is sensitive to the anti-cancer therapy. However, as the treatment replicate, pre-existed subpopulations which are

intrinsically resistant to the drug thrive while subpopulation which was predominant shrink in their proportion. Moreover, there can be appearance of new subpopulation which never existed before, but has resistance to the prior drug. This is mainly due to the genetic evolution of cancer. After the resistant subpopulation increases, the cancer no longer response to the prior drug, and becomes resistant to the specific drug. Furthermore, the proportion of the tumor tissue can migrate to the distant location. In this situation, the metastatic cancer can have very different subpopulational proportion in contrast to the original primary tumor (Figure 1).

In addition to the genomic intra tumor heterogeneity, there are also non-clonal intra tumor heterogeneity. This is mainly explained by functional plasticity and stochastic plasticity. The functional plasticity is the effect due to the cancer communication among tumor microenvironment. Generally, different clonal populations autocrine or paracrine to send signs to each other. Stochastic plasticity mainly accounts the primary tumor heterogeneity with genomic instability[6].

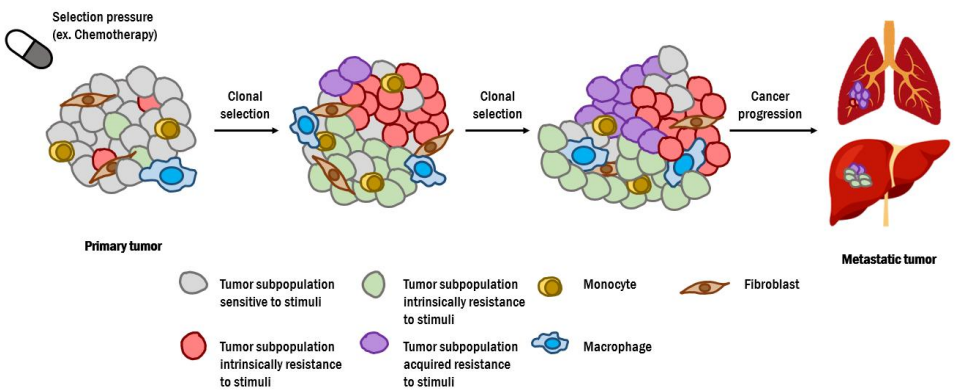

Figure 1. Tumor heterogeneity. In the primary tumor, there were dominant drug sensitive subpopulation, as well as drug resistance subpopulation. This is intratumoral heterogeneity. Throughout the treatment, the clonal selection of thriving drug resistance subpopulation is undergone. After cancer progression, the subpopulational composition is very different from the early stage and metastatic region can even have completely different composition of clonal population.

1.4 Peptide prodrug platform DEVD

To overcome the main hurdle of tumor heterogeneity, there need to be more than specific phenotypic target like tyrosine kinase receptors. Novel concept of targeting apoptosis as "induced phenotype" is suggested to target biological phenomenon rather than specific phenotypic expression[7].

In the apoptotic event, cysteinyl aspartate proteinase family, which is caspase family, is released. There are briefly two main categories of caspase. Pro-apoptotic caspases (caspase-2, 3, 6, 7, 8, 9, 10) are related to cell death signal pathway. Pro-inflammatory caspases (caspase-1, 4, 5, 11, 12) are enzymes which mediates cytokine maturation progress in inflammation situation. In advance, caspases can be further re-organized by its function in initiator caspases (caspase-1, 2, 4, 5, 8, 9, 10, 11, 12) and effector caspases (caspase-3, 6, 7). Effector caspases have short prodomains and work directly at signal transduction in apoptosis execution steps (Table 1).

Function	Mammalian Caspases
Apoptotic Initiator caspases	Caspase-2, 8, 9, 10
Apoptotic Effector caspases	Caspase-3, 6, 7
Inflammatory Initiator caspases	Caspase-1, 4, 5, 11, 12

Table 1. Functional classification of caspases

Apoptosis pathway is divided into extrinsic pathway and intrinsic pathway. In extrinsic pathway, outer death signals such as Fas binds to its receptor, recruit caspase 8 and 10. Subsequently, caspase 3,7 is activated and apoptotic events occur. On the other hand, in intrinsic pathway, apoptotic stimuli, such as DNA damage and stress, is a trigger for initiation. Stimuli makes mitochondira to

release cytochrome c which forms apoptosome. Then, caspase 3,7 is activated in mediation of caspase 9. As shown, varioius kinds of caspases play essential role in apoptosis signal transduction.

Herein, we focused on specific caspase substrate peptides. According to researches caspases matches to peptide substrates which are recognized to specific caspases [8]. Important executioner caspase or effector caspase 3, 7 recognize DEVD peptide as a substrate. Therefore, DEVD peptide could be appropriate peptide for our prodrug (Table 2).

Caspase	Prediction from structure	Peptide substrate
1	WEHD	WEHD
2	DEXD	VDVAD
3	DE(A/V)D	DEVD
6	(L/I/V)E(A/V)D	VEID
7	DE(A/V)D	DEVD
8	(L/I/V)EXD	IETD
9	(L/I/V)EXD	LEHD

Table 2. Peptide substrate for specific caspases.

By using DEVD peptide, we can target apoptosis as induced phenotype, which release caspase-3 to cleave peptide and free drug payload. Using induced phenotype of apoptosis is beneficial in the aspect of recurrent bystander effect. Standard bystander killing effect has its restriction on not effecting overall tumor cell, limiting on very nearby cells of targeted ligand. Recurrent bystander effect model suggests that initial bystander effect activate other prodrugs, results in chain activation through tumor cell. We call it Induced phenotype targeted therapy.

In this model, DEVD peptide link functional moiety and active drug. Functional moiety can give prodrug particular ability such as targeting ability or oral delivery (Figure 2). Not only linking two

parts, DEVD peptide is a caspase 3 cleavable peptide. Exploiting this characteristic, transporting prodrug to one specific phenotypic cell induce apoptosis in particular cell, then leading to activation of prodrug at adjacent cells. Nearby cells which effected by activated prodrug express apoptosis in order, releasing caspase to other adjacent prodrug again and again. Recurring characteristic of this prodrug system can be compared with typical bystander effect, which cannot activate prodrug over and over. This chain like reaction focus on apoptosis as induced phenotype, since caspase 3 from apoptosis could activate the prodrug system.

DEVD peptide prodrug platform is composed of 3 main parts; functional moiety, DEVD peptide, loaded drug. Except DEVD as a linker, functional moiety and loaded drug can be modified in certain purpose. In targeting therapy term, either passive or active targeting ability can be gained through modification of functional moiety.

This peptide prodrug platform benefits in both passive and active targeting. EPR (Extended permeation and retention) effect is a key factor in passive targeting. As shown on the figure, tumor vasculatures become leaky and porous, facilitating transportation of macromolecules. Moreover, lack of effective lymphatic drainage results in prolonged retention of macromolecule. Not only in vasculature characteristics, in systemic term, renal clearance has its limitation on macromolecules. Molecules which have larger size than 40 kDa have low renal clearance leading to extended half-life and emphasized EPR effect.

Albumin binding functional moiety also contribute to passive targeting. Albumin is great carrier protein for numerous evidences. Albumin is soluble, stable in blood pH, biodegradable. Moreover, tumor endothelial cells have gp 60 receptor which specifically intake albumin, interacting with SPARC protein on tumor cells, results in tumor preference of albumin. As albumin is one of blood protein, albmin has comparatively low toxicity than other synthetic carrier protein. Since albumin is blood protein, binding with albumin can prolong half life of drugs. Albumin bound drugs can be targeted

in passive way via SPARC protein interaction. To use albumin for prodrug carrier protein, selection over albumin was done. Serum albumin is superior than synthesized albumin in that serum albumin is not pathogenic, simple in production and easily analyzable.

Modification in Fuctional moiety in active targeting term can be suggested with RGD peptide. RGD peptide targets Integrin $\propto_V \beta_3$, which numerously expresses in tumor endothelial cell, playing important role in Metastasis. Integrin $\propto_V \beta_3$ is related with mechanism of breaking down collagen in extracellular matrix through MMP2. As a result, it is associated with tumor migration induction, metastasis, angiogenesis. Thus, RGD peptide moiety can be used to target tumor endothelial cells in metastatic cancer. Furthermore, via switching therapy, RGD moiety platform can trigger initial apoptosis instead of radiation, eliminating the disadvantage of the radiation therapy.

Not only exploiting integrin $\propto_V \beta_3$, ligand-receptor reaction, antigen-antibody reaction can be exploited as well. Other examples can be angiopep-2 which binds to LRP1.

In peptide prodrug platform, not only functional moiety, but also active drug can be altered. The loading drug should be nonselective cytotoxic drug that can be diffused to cell via simple diffusion. In our lab Doxorubicin, MMAE, Docetaxel were selected as candidates.

Figure 2. Concept of induced phenotype targeted therapy(IPTT)

1.4.1 Previous IPTT with radiation

To initiate the activation of prodrug at certain lesion, radiation therapy has been suggested to trigger apoptosis, since radiation can be used at specific region. After initial apoptosis is induced by

radiation, caspase 3 activates the prodrug. Activated drug cause apoptosis, resulting caspase 3 expression which can activate nearby prodrugs effecting to adjacent tumor cells. In conclusion, after first strike with radiation to patients, injecting prodrug can efficiently have impact on targeted tumor cell marked by radiation. Although this recurrent bystander killing effect poses way to overcome heterogeneity, radiation as a trigger of treatment is critical point, since it has several disadvantages. Patients have maximum limits of getting radiation therapy over lifetime. Additionally, there are adverse reaction of radiation such as skin damage, GI problem, hair loss.

1.4.2 Targeted therapy triggered prodrug for metastatic tumor

To further expand DEVD prodrug system to the metastatic tumor, the radiation cannot be used. Since radiation only targets specific lesion, trigger that induce apoptosis at every metastatic lesion is required. Metastatic cancers invade and grow in multiple locations. Therefore, we screened the new trigger for DEVD prodrug platform.

The new trigger for prodrug system, targeting agent was suggested. Even though classical targeted therapy in monotherapy regimen met hurdle in drug resistance developed in tumor tissue, the targeted therapy for DEVD prodrug system was used only to induce initial apoptosis, not eradicating cancer. Since targeted therapy can attack cancer cells without harming normal tissue unlike conventional chemotherapy, it could selectively induce apoptosis at tumor tissue. It is especially beneficial in metastatic cancer, because only treating targeted therapy can induce apoptosis in all metastatic lesion. However, not all targeted therapy can be exploited in our DEVD prodrug system. Since DEVD peptide should be cleaved by caspase-3 released by apoptosis of cancer cells,

cytotoxic agents which induce apoptosis and release caspase-3 is necessary. Thus, it is necessary to screen cytotoxic drug which efficiently induce apoptosis and release caspase-3.

1.5 Metastatic pancreatic cancer

Over many kind of cancers, pancreatic cancer is one of the most fatal cancer for mankind. The high fatality of pancreatic cancer is mainly due to the late diagnosis time. Pancreatic cancer is called silent cancer, since it has almost no symptom until it reaches the late stage of metastasis. According to the statistics, 52% of the pancreatic cancer is get diagnosed at metastatic stage, showing only 2.7% of 5-year survival, the lowest comparing to the 34.3% 5-year survival for localized pancreatic cancer diagnosis and 11.5% of 5-year survival for regional pancreatic cancer at diagnosis[9] (Figure 3). Therefore, the cure for the metastatic pancreatic cancer is critical.

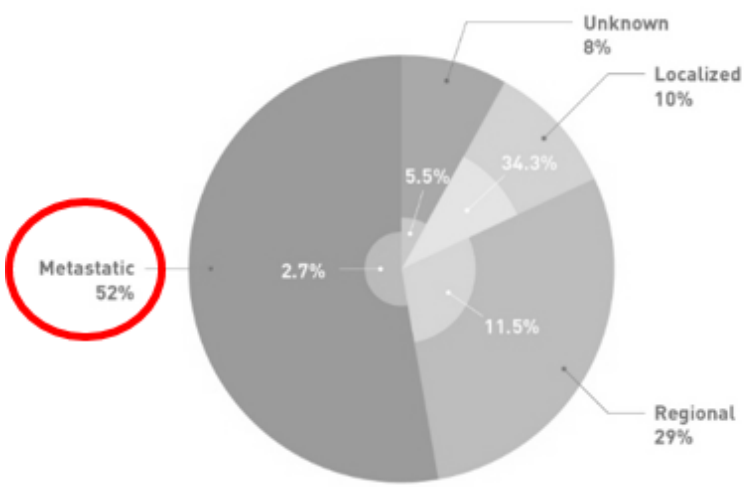

Figure 3. Incidence and 5-year survival for pancreatic cancer by stage at diagnosis

Among the metastasis site of the pancreatic cancer, around 80% of metastatic site involved the liver site metastasis. Statistically the origin of liver metastasis is mainly from colorectal cancer or pancreatic cancer[10]. 46.1 % of liver metastasis derived from colorectal adenocarcinoma and 10.1 % of liver metastasis derived from pancreatic adenocarcinoma (Figure 4). Liver is originally main target of metastasis with lung since it has thin capillary blood vessels. Moreover, in physical hemodynamic hypothesis, the high probability of liver metastasis from gastrointestinal cancer is due to the hepatic blood vessel anatomy. Hepatic blood vessel is composed of hepatic triad; bile duct, hepatic artery, and portal vein. Bile duct carries bile through liver. Hepatic artery supplies oxygen rich blood to the liver from heart. Portal vein is sprouted from the gastrointestinal tract, carries nutrient absorbed from intestinal lumen. Portal vein origins from superior mesenteric vein from jejunum and ileum, and inferior mesenteric vein from descending and sigmoidal colon [11]. Mesenteric veins merges with gastric vein and veins from pancreas and spleen. Portal vein finally enters liver. In gastrointestinal cancer patients, cancer detached from primary site flow through portal vein and enters liver easily. Hepatic artery and portal vein integrate into blood stream called arteriolar inlet through peribiliary vascular plexus. Also portal vein itself enters the liver solely through portal vein inlet.

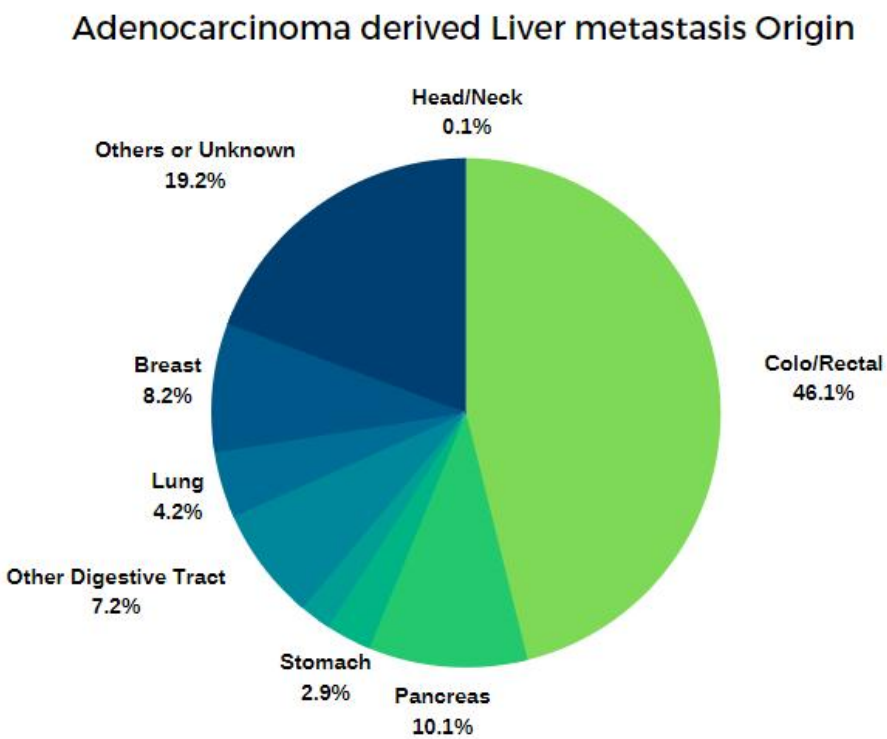

Figure 4. Adenocarcinoma derived liver metastasis origin

1.6 Olaparib at pancreatic cancer

Herein this research has selected Olaparib as triggering targeted therapy. Olaparib is small molecule targeted therapy which inhibits PARP protein. Olaparib was first approved for ovarian and breast cancer. Olaparib works in BRCA gene mutant patient. Human DNA repair system is composed of single strand break and double strand break. Especially in the double strand break, BRCA and PARP plays key role in the pathway. Thus BRCA mutant patient results in defect in DNA repair system when they are treated with PARP inhibitor. If their BRCA and PARP are both inhibited, cancer cell falls into apoptosis. Therefore, Olaparib is only applied to BRCA mutant patients [12]. Since Olaparib is only working in BRCA mutant patients, it is used with co-diagnosis system, which is BRCAnalysis. BRCAnalysis co-diagnosis system evaluate the

patient genotype to whether it is suitable to use PARP inhibitor such as Olaparib.

Recently, Olaparib has expanded its indication to maintenance therapy in metastatic pancreatic cancer patients. This expansion of indication was based on the POLO trial. The POLO trial is the clinical trial which compare the Olaparib effect on metastatic pancreatic cancer patients, who has taken prior 16 weeks or more platinum based chemotherapy. 154 patients were randomized to either receive Olaparib or placebo. In the results, Olaparib treated group showed 47% reduction in relative risk of disease progression or death. Also compared to the 3.8 months median Progression Free Survival (PFS) in control group, Olaparib treated group showed 7.4 months of median PFS. Also Olaparib nearly doubled Overall Response Rate (ORR) from 12% in control group to 23% in test group. More specifically, estimated PFS with Olaparib versus placebo at 6 months was 53% versus 23% and at 1 year was 34% versus 15% and at 2 years was 22% versus 10% [13]. In sum, there was recent rationale for using Olaparib in metastatic pancreatic cancer.

1.7 Oral metronomic maintenance therapy for metastatic patients

Conventional regimen of anti-cancer drugs was administered in maximum-tolerable dose schedule. Maximum-tolerable dose schedule administers anti-cancer drug in maximum dose which patients can tolerate without severe dose limiting toxicity. In such regimen, first line therapy was given and rest period is required for patients to recover from anti-cancer drug's systemic toxicity. After the resting period, there is evaluation of cancer progression and if there are progression in disease, there will be second line therapy of maximum-tolerable dose drug administration. The next clinical regimen concept was maintenance therapy, which provides patients

maintenance therapy after the first treatment with lower dose. This delays the administration of second treatment which can protect patients from being exposed to variety of anti-cancer drug in short period. The final concept I want to introduce is metronomic maintenance chemotherapy. This is frequent dosing system with lower dose which fits in the therapeutic window of specific drug. By frequently dosing, metronomic maintenance chemotherapy benefits in 3 ways. It directly kills cancer cells by suitable concentration of anti-cancer drug itself. Also it inhibits tumor angiogenesis which is essential in tumor proliferation. Moreover, it has much lower immune toxicity than maximum-tolerable dose regimen. Rather, it even has immune stimulating effect, which positively affect anti-cancer effect [14]. By using frequent dosing, metronomic maintenance chemotherapy prefers oral drugs for the ease of administration. Also metronomic chemotherapy eventually lowers probability of side effect expression.

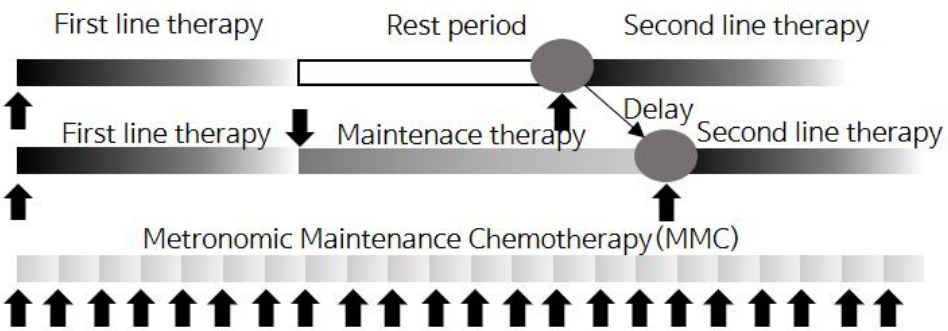

Figure 5. Concept of metronomic maintenance chemotherapy (MMC)

1.8 Oral drug delivery using bile acid derivatives

To make previously mentioned DEVD peptide to oral drugs, this research has exploited bile acid derivatives as oral drug delivery system. Bile acid derivatives were researched for oral drug delivery [15], [16]. Bile acid derivatives are recycled through enterohepatic

circulation. Their absorption mainly occurs in the ileum and the mechanism of absorption is various including bile acid transporter mediated endocytosis and paracellular permeation. Among many bile acid derivatives, deoxycholic acid was selected and conjugated with lysine, which exhibits positive charge. This positive charge forms electrostatic complex with glutamate and aspartate from DEVD peptide linker in the prodrug. Complexed deoxycholic acid lysine conjugate induce oral absorption of DEVD prodrug complex.

1.9 OPD3 system

The OPD3 main concept is combination regimen with Olaparib. OPD3 and Olaparib both are administered orally in metronomic schedule. Orally delivered DEVD prodrug OPD3 is absorbed in the ileum part of the gastrointestinal tract. Absorbed OPD3 and Olaparib reaches target organ. The primary apoptosis is induced by Olaparib, which is targeted therapy for BRCA mutated cancer cells. Olaparib induces apoptosis and apoptotic cells release caspase-3. Released caspase-3 cleaves DEVD peptide from the prodrug. Then the active drug docetaxel is relieved and work in adjacent cancer cells including BRCA wild type cells which initially cannot induce apoptosis by treating Olaparib. Such secondary apoptosis also cause release of caspase-3 and activate further release of active docetaxel to nearby cancer cells. Finally, recurrent bystander effect activate prodrug in targeted region where cancer cell is initially targeted by Olaparib.

OPD3 strategy finally targets metastatic pancreatic cancer which frequently express as liver metastasis. Orally delivered OPD3 and Olaparib is expected to directly delivered to the target organ liver. Also in the metronomic way, metastatic cancer is expected to be well controlled. Ultimate goal of this research is identifying anti-cancer effect of OPD3 and Olaparib in combination regimen in BRCA mutant pancreatic tumor model and especially in

the liver metastasis model.

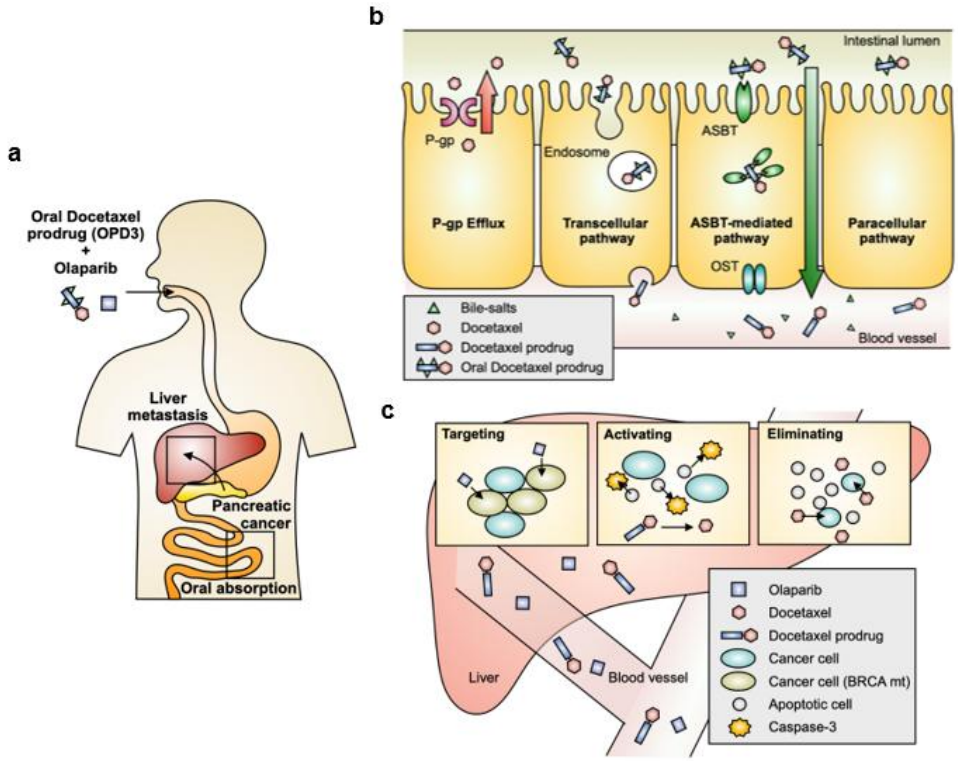

Figure 6. Prodrug and oral delivery concept of OPD3

Chapter 2. Materials and methods

2.1 Materials

AcK(Alloc)GD(All)E(All)VD(All)-OH peptide was purchased from GL Biochem (Shanghai, China). Docetaxel was purchased from Medchem Express (Monmouth, NJ). p -Aminobenzyl alcohol, Dimethylformamide, N,N-Diisopropylethylamine, 4-Dimethylaminopyridine, Dichloromethane, Acetic acid, and N-methyl morphorline were purchased from Sigma Aldrich (St. Louis, MO). 2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline, bis(p-nitrophenyl) carbonate, and Tetrakis (triphenylphosphine)-palladium(0) were purchased from TCI (Tokyo, Japan). All other solvents were purchased from Honeywell (Seoul, South Korea).

Deoxycholic acid (DOCA), Ethyl chloroformate, N-methyl morpholine, Acetyl chloride, sodium bicarbonate were purchased from Sigma Aldrich, and Lys(Boc)OMe-HCl was purchased from GL biochem.

DMSO, Glucose, HEPES, Actinomycin D, Chlorpromazine, Brefeldin A, Genistein, Amiloride, Cyclosporin A were puchased from Sigma Aldrich. 20X PBS was purchased from Biosesang (Seongnam, Korea). HBSS was purchased from Corning (Manassas, VA).

Olaparib was purchased from Medchem Express (Monmouth, NJ).

Human recombinant caspase-3 was purchased from R&D systems (Minneapolis, MN)

2.2 Cell lines

Caco-2, Capan-1 cells were purchased from Korea Cell Line Bank (Seoul, South Korea). Miapaca-2 cell was a kind gift of Asan medical center (Seoul, South Korea). Capan-1-luc2 cell was

transfected by Yonsei University College of Pharmacy (Incheon, South Korea). Caco-2 and Miapaca-2 were cultured in Dulbecco's modified eagle medium (DMEM; Corning) added with 10% fetal bovine serum (FBS; Gibco), 1% non-essential amino acid (Gibco), and 1% penicillin-streptomycin (Gibco). Capan-1 and Capan-1-luc2 were cultured in Rosewell Park Menorial Institue - 1640 (RPMI-1640; Corning) supplemented with 10% FBS and 1% antibiotics mentioned above. The cells were under control in humidified 5% CO2 atmosphere at 37 °C.

2.3 Synthesis of KGDEVD-Docetaxel and DCK

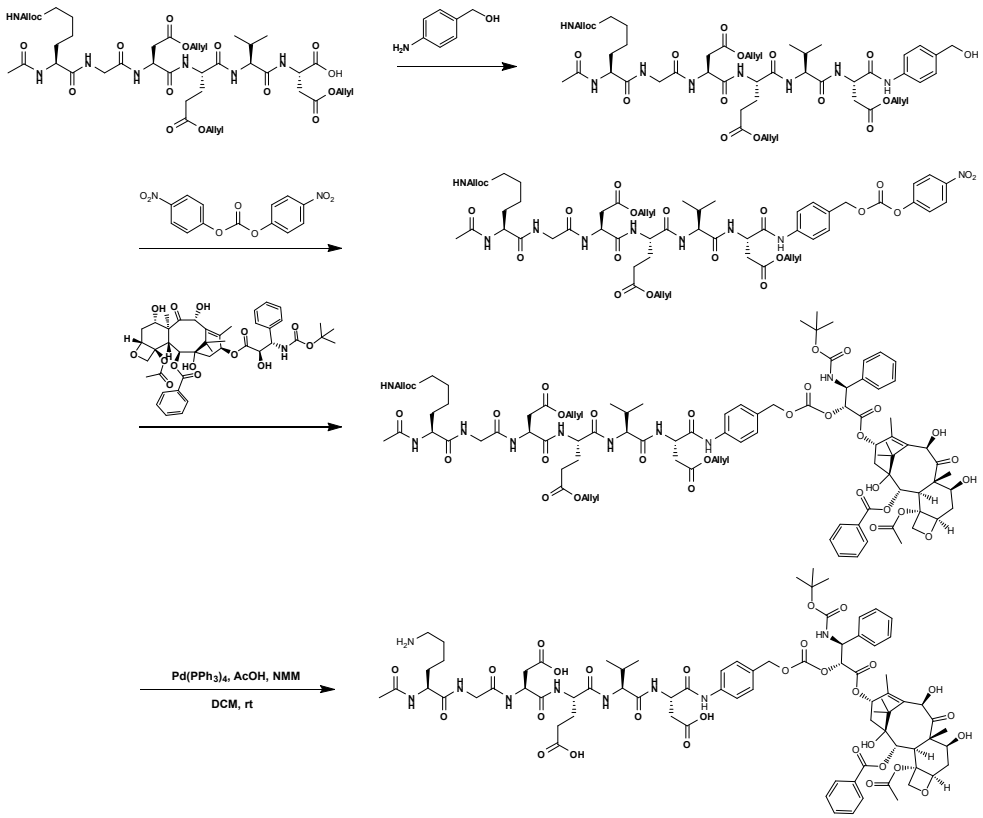

Figure 7. Synthesis scheme of KGDEVD-Docetaxel

AcK(Alloc)GD(All)E(All)VD(All)-OH (500mg), p-aminobenzyl alcohol (138mg), and EEDQ (278mg) were dissolved in anhydrous DMF (25mL). The mixture was reacted for 16 hours

at room temperature. After 16 hours of reaction, the completion of reaction was identified by checking HPLC (Agilent Infinity series 1260). By the end of the reaction, the mixture was evaporated to the volume of under 5mL. The obtained product is then precipitated in cold ether (120mL). Over 4hours of precipitation, the precipitant and ether is filtered through membrane filter (JHWP PTFE hydrophilic 0.45nm filter). The collected precipitate was dried in vacuo to obtain AcK(Alloc)GD(All)E(All)VD(All)-PABA. AcK(Alloc)GD(All)E(All)VD(All)-PABA (520 mg), bis(p-nitrophenyl)carbonate (780 mg)were dissolved in anhydrous DMF(25mL). Then DIPEA (285uL) was added dropwise to the mixture. The mixture was reacted for 16 hours at room temperature. The completion of reaction was identified by checking HPLC. By the end of the reaction, the mixture was evaporated to the volume of under 5mL. The obtained product is then precipitated in cold ether (120mL). Over 4 hours of precipitation, the precipitant and ether is filtered through membrane filter (JHWP PTFE hydrophilic 0.45nm filter). The collected precipitate was dried in vacuo to obtain AcK(Alloc)GD(All)E(All)VD(All)-PABC. AcK(Alloc)GD(All)E(All)VD(All)-PABC (467.8mg), Docetaxel (384.7mg), and DMAP (145.1mg) were dissolved in anhydrous DMF (15mL). Reaction underwent for 16 hours at room temperature. The completion of reaction was identified by checking HPLC. By the end of the reaction, the mixture was evaporated to the volume of under 5mL. The obtained product is then precipitated in cold ether (120mL). Over 4hours of precipitation, the precipitant and ether is filtered through membrane filter (JHWP PTFE hydrophilic 0.45nm filter). The collected precipitate was dried in vacuo to obtain AcK(Alloc)GD(All)E(All)VD(All)-PABC-DCX. AcK(Alloc)GD(All)E(All)VD(All)-PABC-DCX (558mg) was dissolved in dichloromethane, Acetic acid, and N-methyl morpholine mixture with ratio of 37:2:1. Pd(PPh3)4 (1047.6 mg) was dissolved in dichloromethane and added slowly to the mixture. The deprotection was reacted for 4 hours at room temperature. Deprotected AcKGDEVD-PABC-DCX precipitate was filtered using

membrane filter (JHWP PTFE hydrophilic 0.45nm filter). The precipitate was washed with dichloromethane and ether in order. The precipitate was dried in vacuo to earn AcKGDEVD-PABC-DCX powder. For purification, AcKGDEVD-PABC-DCX was dissolved in 10% Acetonitrile and 4% Acetic acid solution to be injected to semi-preparative reverse-phase HPLC (Shimadzu, Kyoto, Japan) using an octadecylsilyl (ODS-A) 5 μm semi-preparative column (150 x30mm; YMC, Dinslaken, Germany). Acetonitrile with 1% acetic acid was used as solvent. Sample was separated at the condition of isocratic 38% acetonitrile with flow rate of 5mL/min detected with VWD detector of 254nm. Collected fractions were evaporated and lyophilized by freeze dryer.

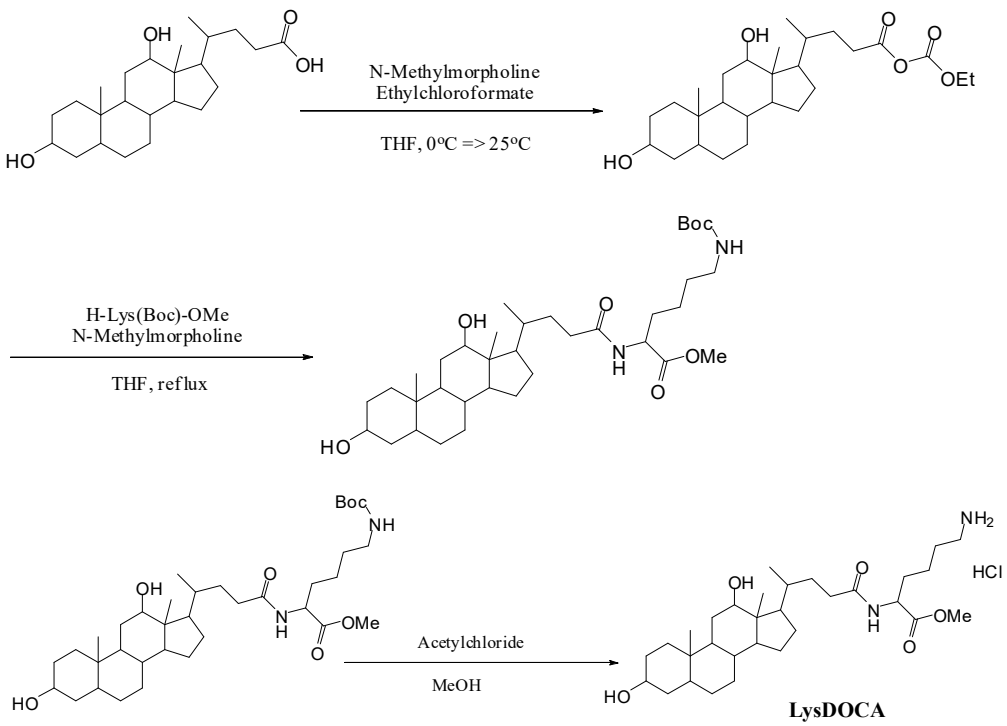

Figure 8. Synthesis scheme of Deoxycholic acid lysine

DCK was synthesized from Deoxycholic acid following previously published protocol[17]. To briefly mention, Deoxycholic acid (40g) was dissolved in anhydrous THF (1.5L) and purged with N2 gas. After cooling at 0 ˚C, add Ethylchloroformate (9.7ml) and

4-Methyl morpholine (11.2mL) in order. Then, it is reacted at 0 °C for 30minutes and followed by 2 hours room temperature reaction. 4-Methyl morpholine (11.2mL) is added again and pour Lys (Boc) OME-HCl (30.2g) to the flask. After refluxing from 50 °C to 70 °C for 2 hours, react overnight. Then filter using filter agent (Celite 545, Sigma 419931) and evaporate sample. Purification was done by using open column (Column mobile phase CHCl3 : MeOH = 20 :1 v/v%, TLC mobile phase = CHCl3 : MeOH = 9:1 v/v%, staining with PMA). After purification, Acetyl chloride (44.76mL) with MeOH (628mL) is prepared at 0 °C. Stirred for 30minutes, Lys(Boc)DOCA (40g) is added. React overnight at room temperature. Evaporate the sample and dissolve with 400mL of DW and wash with 400mL chloroform. The final sample was obtained by lyophilizing DW layer.

2.4 Preparation and formulation of DEVD-S-Docetaxel/DCK complex

KGDEVD-Docetaxel (10.6mg) and DCK (3 equivalent amount, 11.024mg) was dissolved in DDW (5mL). Using pH meter, slowly titrate mixture solution with 0.1N NaHCO3. Add 0.1N NaHCO3 until pH reaches 7. Centrifuge at 3000rpm for 10minutes and aspirate supernatant. Wash with DDW and centrifuge 3 times. Collected final precipitate was dried in vacuo.

For the administration in the in vivo studies, 5% labrasol (Gattefosse, St. Priest, France) and 2.16 mg/kg poloxamer were added as solubilizers for liquid formulation.

2.5 Characterization of complex

The physical complex of DEVD-Docetaxel and DCK was confirmed by DSC thermogram with a Discovery DSC instrument

(TA Instruments, New Castle, DE). The complex was formed in water and dried powder sample was evaluated using a heating rate of 10 °C/min and the temperature was increased up to 300 °C.

2.6. Cytotoxicity assay

The CCK-8 kit (Dojindo, CK04) was exploited to assess the toxicity of the Olaparib, Docetaxel, and DEVD-Docetaxel. Each cell lines (Capan1, Miapaca-2) were planted in a 96-well plate and incubated at 37 °C with 5% CO2 for 24hr. Then, the cells were treated with pre-determined concentration of each drugs. The drug was treated 2 days for Docetaxel and DEVD-Docetaxel and 3 days for Olaparib. Lastly, the CCK-8 reagent was added and incubated for 1 to 2 hours. The cell viability is calculated as following equation:

Cell Viability (%) = (Absorbance of sample cells – Absorbance of Blank well) / (Absorbance of Control cells – Absorbance of Blank well) * 100

2.7 Caspase activity assay

The caspase-3 assay kit from abcam was used to quantify the released caspase-3 from Olaparib treated Capan-1 cell line according to the different concentration. Capan-1 cell was cultured on the 100 pie dish. After 1 day of incubation, Olaparib was treated 50, 10, 1uM in media with 1% DMSO. The plates were incubated for 3days. Cells including control group were scraped from the culture plate, suspended in the cell lysis buffer. After quantifying the protein by the BCA protein assay kit (Thermoscientific, 23227), the samples were separated using lysis buffer by equalizing the protein amount. Samples were reacted with reaction buffer with 10mM DTT and 4mM DEVD-pNA substrate. After 1hours of incubation the OD value was measured by multi plate reader with wavelength of

405nm. The caspase-3 amount was compared via the fold-increase to the control group caspase-3 amount.

2.8 Caspase-3 mediated Docetaxel release

OPD3 was cleaved by caspase-3 1ug/ml treatment. The cleaved Docetaxel was analyzed by HPLC peak detected at 254nm wavelength. Mobile phase was acetonitrile with 0.1% trifluoroacetic acid. The gradient was 10% mobile phase from 10minutes to 90% mobile phase in 25 minutes. Accurate amount of the Docetaxel was calculated through calibration curve of Docetaxel in the same analytic condition. Docetaxel calibration curve was obtained with concentration of 25, 50, 125, 250, 500 ug/ml Docetaxel. Caspase-3 treated OPD3 50ug/ml was analyzed at time point of 2 and 4 hours after treatment. Based on the peak area, docetaxel concentration was calculated.

Caspase-3 cleaved OPD3 was also analyzed through LC/MS (Ultimate 3000, Thermoscientific, USA). Agilent Eclips C18 column (2.1 mm X50 mm, 1.8um) was used. Mobile phase was 0.1% formic acid in acetonitrile. The gradient of mobile phase was increased from 10% at 1 minute time point and reached 100% at 6 minutes time point. The retention time point of Docetaxel lies at 4.95 minutes. MS instrument of Triple TOF 5600+ (AB Sciex, USA) is operated to analyze the amount of Docetaxel. Electrospray Ionization (ESI) was used as ionization source. 250ng/ml OPD3 was treated with 1ug/ml Caspase-3 in 2 hours and analyzed to earn docetaxel amount from sample. Upon the calculated docetaxel concentration from known OPD3 concentration, the complex binding ratio was concluded.

2.9 Determination of apparent permeability in Caco-2 cells

Caco-2 cells cultured in DMEM with 10% FBS, 1% NEAA and

1% antibiotics were seeded over the Transwell (Corning, 3460). The cells were incubated in a 37 °C incubator with 5% CO2 for over 21 days to completely form monolayer and differentiated morphology. Media of apical and basolateral side was replaced every other day. For the Caco-2 permeability assay, the media from apical and basolateral side was exchanged to HBSS solution with 5mM of Glucose and 5mM of HEPES. Donor solution was prepared with Docetaxel, KGDEVD-Docetaxel, and OPD3 in concentration of 40ug/ml Docetaxel equivalent; according to the molecular weight, 40ug/ml of Docetaxel, 81.3ug/ml KGDEVD-Docetaxel, 161ug/ml OPD3 respectively were dissolved in HBSS with 1% DMSO. The HBSS solution from the basolateral chamber was obtained at the pre-determined time points (2, 2.5, 3, 3.5, 4 hours). For the quantification of the substances, every sample was treated with Caspase-3 1ug/ml for 2hours to release free docetaxel. The freed docetaxel from KGDEVD-Docetaxel and OPD3 then could be detected using LC/MS. Accumulative transported amount and permeability was calculated. Apparent permeability was calculated as

$$Papp = dQ/dt * 1/A * C0$$

2.10 Absorption mechanism study in Caco-2 cells

Caco-2 cells cultured in DMEM with 10% FBS and 1% antibiotics were seeded over the Transwell (Corning, 3460). The cells were incubated in a 37 °C incubator with 5% CO2 for over 21 days to completely form monolayer and differentiated morphology. Media of apical and basolateral side was replaced every other day. For the mechanism study, the media from apical and basolateral side was exchanged to HBSS solution with 5mM of Glucose and 5mM of HEPES. Donor solution was prepared with OPD3 in concentration of 40ug/ml Docetaxel equivalent; according to the molecular weight, 161ug/ml OPD3 respectively were dissolved in

HBSS with 1% DMSO. The HBSS solution from the basolateral chamber was obtained at the pre-determined time points (2, 2.5, 3, 3.5, 4 hours). In addition to the OPD3, 6 different absorption mechanism inhibitors are added to each group with 3 independent samples. Also for assessing P-gp effect, Cyclosporin A treated to Docetaxel group was added. 6 absorption inhibitors Actinomycin D, Chlorpromazine, Brefeldin A, Genistein, Amiloride, Cyclosporin A are treated to each group in 3.2uM, 32uM, 90uM, 0.1mM, 0.1mM, 10uM respectively. Actinomycin D, Chlorpromazine, Brefeldin A, Genistein, Amiloride, Cyclosporin A are inhibiting Bile acid transporter, Clathrin-mediated endocytosis, ER/Golgi pathway, Caveola-mediated pathway, macropinocytosis, P-gp transporter respectively. Samples from each groups are treated with Casspase-3 1ug/ml for 2hours to cleave DEVD peptide from Docetaxel. Treated samples were analyzed by LC/MS in same condition used over permeability assay. Apparent permeability was calculated and compared to the non-treated control group.

2.11 Pharmacokinetic study

For the pharmacokinetic study, 7-weeks old male SD rats were purchased from Orient Bio Inc (Seoul, Korea). Rats were fasted 24 hours before administrating oral drug. Docetaxel was given intravenously 5mg/kg and orally 20mg/kg. OPD3 was given intravenously 5mg/kg and orally 20mg/kg. Each group had 4 rats for studying. Docetaxel was formulated as 5% DMSO, 30% PEG 300, 5% Tween 80 and distilled water. OPD3 was formulated as 5% labrasol and 2.16 mg/kg poloxamer in PBS. Before orally administering the drugs, 400ul of 5% NaHCO3 was administered to neutralize the gastric acidity. The predetermined time points for intravenous injection group were 5, 15, 30 minutes, 1, 2, 4, 6, 10 hours. The predetermined time points for oral injection group were 15, 30 minutes, 1, 2, 3, 4, 6, 10 hours. Collected blood samples were mixed with citric acid 50ul. Blood samples were centrifuged in 6000rpm 5 minutes. The supernatant was collected as analytic

samples. Standard sample was prepared with Docetaxel in final concentration of 1, 2, 10, 20, 100, 200, 2000 ng/ml in blank plasma with 10% methanol with caspase buffer. Each blood samples were treated with caspase buffer (6.25 ug/ml) for 1hour in heat bath maintaining 37 ˚C. Then internal standard of 2.5ug/ml paclitaxel in methanol 100ul was added. Plasma proteins were precipitated by adding 500ul of methanol. Then docetaxel was extracted by adding 500ul of diethyl ether. The mixture was centrifuged in 10000rpm for 5 minutes. The supernatant 800ul was obtained for docetaxel sample. The sample was dried in speed-vac in condition of 40 ˚C for 3 hours. Dried solid samples were reconstituted with 100ul methanol before analysis. Reconstituted samples were centrifuged in 10000 rpm for 5 minutes and supernatant 80 ul was obtained for analytic sample. Samples were analyzed by LC/MSMS spectrometer (TSQ Altis, Thermo Fisher Scientific, USA). The docetaxel concentration was calculated from calibration curve. Pharmacokinetic parameters including half-life, T max, C max, AUC last, AUC infinite, mean residual time, and bioavailability are calculated using Phoenix Winnonlin program.

2.12 Tumor growth suppression study

To study tumor growth inhibition effect of Olaparib and OPD3 in vivo, BRCA mutant human pancreatic cancer cell line Capan-1 and BRCA wild type human pancreatic cancer cell line Mia paca-2 cell line were selected as xenograft model. Capan-1 was cultured in RPMI with 10% FBS and 1% antibiotics. Miapaca-2 was cultured in DMEM with 10% FBS and 1% antibiotics. For the Capan-1 and Miapaca-2 Xenograft model, 7-weeks old female Balb/c nude mice of average body weight 20-25g were purchased from Orient Bio Inc (Seoul, Korea). 1 x 107 Capan-1 cells were subcutaneously seeded into the left back side of the mice. While inoculating the cells, mice are anesthetized by intraperitoneal injection of 2,2,2-

Tribromoethanol diluted in the distilled water and 2-Methyl-2-butanol. The mice were assigned in random order and monitored until the tumor volume reached 75 -100mm3. In the Capan-1 xenograft model, 5 groups are control, Olaparib 50mg/kg, OPD3 5mg/kg, OPD3 5mg/kg and Olaparib combination, OPD3 10mg/kg and Olaparib combination. Olaparib was given daily in 50mg/kg per oral (po). In the Miapaca-2 xenograft model, 4groups except OPD3 10mg/kg and Olaparib combination was constructed. OPD3 was administered at a dose of 5mg/kg or 10mg/kg per oral (po) daily. Olaparib was formulated in 5% DMSO, 10% cyclodextrin and PBS. Mice were treated for 28 days. During monitoring, tumor volume and body weight were measured every other day. Tumor volume was calculated as [(Length x Width2) x0.5]. After administering drugs and monitoring for 28 days, mice were sacrificed and tumor tissue is obtained. Tumor weight and picture was taken after sacrifice.

2.13 Liver metastasis model Establishment

For the establishment of liver metastasis model, this research has taken two different liver metastasis model. First, we established portal vein injection liver metastasis model. Balb/c nude mice were anesthetized by intraperitoneal injection of 2,2,2-Tribromoethanol diluted in the distilled water and 2-Methyl-2-butanol. With the surgical scissor, the abdomen region was opened. Intestines were carefully put out on the wet gauze soaked by normal saline. Find portal vein and fasten portal vein with tweezers for injection of cells. Cells were injected with 30G 0.5ml insulin syringe. After injecting cells, to avoid bleeding, give pressure on sterile gauze or cotton swab on the injection site until the bleeding ends. Put intestines carefully back to the original location and suture the opened abdomen with sterile 4-0 vicryl suture.

Another model was intra-splenic injection model. Balb/c nude mice were anesthetized in same condition. Middle left back side of

abdomen was opened by surgical scissor, and spleen was exposed outside. Cells were injected directly to the spleen and wait for 5 minutes for the circulation of cells. Then cut off half of the spleen including the cell injection site, preventing tumor outgrowth in the spleen. Hemostatic gauze or cotton swab was used to stop bleeding after excision. After stop of bleeding, spleen was set back to the original location and peritoneal membrane was sutured. And skin was nipped by surgical clips.

2.14 Statistical analysis

Data from in vitro experiments are indicated as mean ± standard deviation of results obtained from three independent experiments unless presented in other way. Data from in vivo experiments are indicated as mean ± standard error of mean of results obtained from previously mentioned number of samples used in each experiments. Analysis of variance (ANOVA) was exploited to show statistical significance between three or more groups. P-values of <0.05 are considered statistically significant.

Chapter 3. Results and discussion

3.1. Synthesis of KGDEVD-Docetaxel and DCK

KGDEVD-Docetaxel was synthesized from protected KGDEVD peptide. PABC linker was ligated to the protected peptide and docetaxel was loaded to the peptide and linker. Then deprotection was done. Total 5 batches of KGDEVD-Docetaxel were synthesized for the research. Step yield and accumulative yield are shown with the Table 3. At the last step, semi-preparative HPLC was used to purify the KGDEVD-Docetaxel product. Final purity was maintained over 98% in single HPLC peak.

		Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
Step1	Synthesis of AcK(Alloc)GD(All)E(All)VD(All)-PABA					
	Step Yield (%)	92.27	95.0212024	96.20395	106.2959	91.47962
	Accumulative Yield (%)	92.27	95.0212024	96.20395	106.2959	91.47962
Step2	Synthesis of AcK(Alloc)GD(All)E(All)VD(All)-PABC					
	Step Yield (%)	77.4	79.1833907	76.92695	74.10097	74.59759
	Accumulative Yield (%)	71.4	75.2410099	74.00676	78.76629	68.24159
Step3	Synthesis of AcK(Alloc)GD(All)E(All)VD(All)-PABC-DCX					
	Step Yield (%)	75.5	67.6473602	80.69705	66.32649	89.38547
	Accumulative Yield (%)	53.9	50.9	59.72127	52.24292	60.99806
Step4	Deprotection of AcK(Alloc)GD(All)E(All)VD(All)-PABC-DCX					
	Step Yield (%)	73.5	80.9831449	84.12976	85.67187	69.80994
	Accumulative Yield (%)	39.6	41.229	50.24336	44.75748	42.58271
Step5	Preparative LC					
	Step Yield (%)	17	31.9883196	26.37392	24.12359	35.59192
	Accumulative Yield (%)	6.7	13.2	13.25114	10.79711	15.15601
	Purity (%)	98.3	98.8	99	98.2	99.1

Table 3. Synthesis scale of KGDEVD-Docetaxel

The HPLC peak for each step product was shown with Figure 9. Final product showed sharp single peak with high purity of over 98%. The final product was also identified with mass

spectrometry. The molecular weight of 1642.69 was identified with mass spectrometry, showing the mass to charge ratio of 1643 in $(M+H)^+$ form.

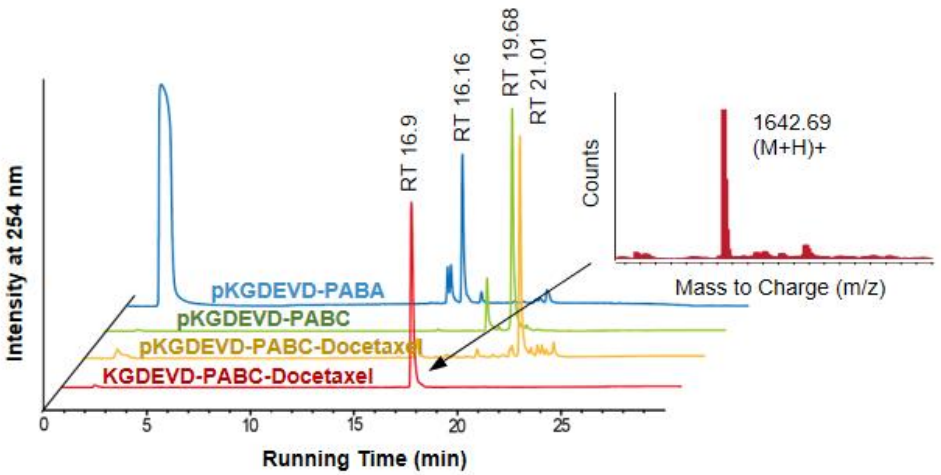

Figure 9. Synthesis of KGDEVD-Docetaxel prodrug and its HPLC, MS data

DCK was synthesized by the method which mentioned previously. Final product of DCK salt was lyophilized and stored at 4 ˚C.

3.2 Preparation and formulation of DEVD-S-Docetaxel/DCK complex

To obtain DEVD-Docetaxel/DCK complex, which is OPD3, DEVD-Docetaxel and DCK was dissolved in water and mixed. The mixture was then neutralized with sodium bicarbonate solution. Since the binding sites for the positive charge of DCK are 3 in DEVD peptide, Aspartate and Glutamate, 3.1 equivalent of DCK was prepared for the mixture. The bounding site is shown with the Figure 10. The binding is based on the electrostatic force between positive charge of lysine and negative charge of aspartate and

glutamate. The initial pH of mixture was acidic, but with the addition of sodium bicarbonate, lysine part of DCK can be protonated and become positively charged, so that the electrostatic complex can be formed.

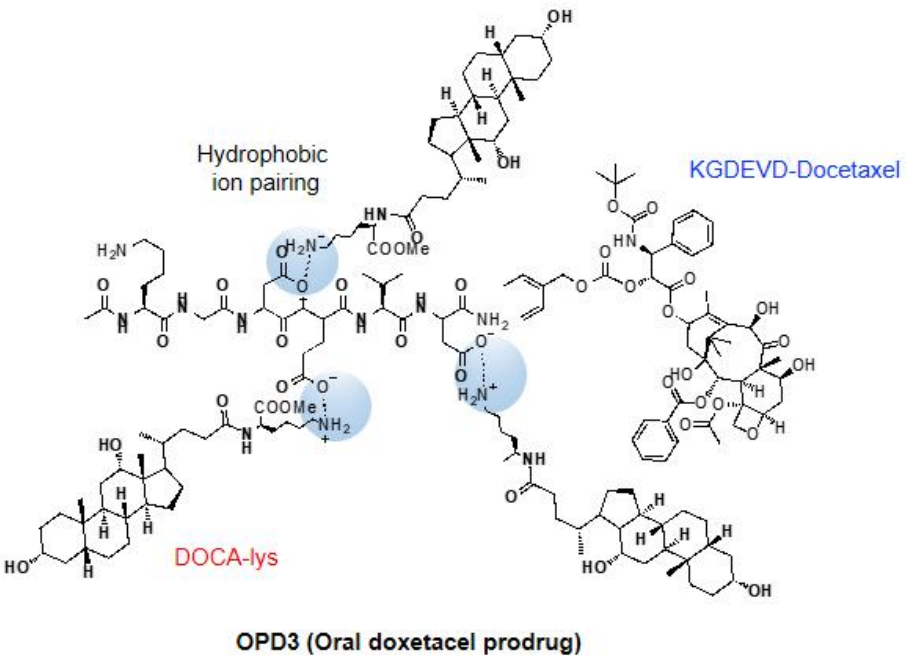

Figure 10. Physicochemical complex of OPD3

OPD3 was hydrophobic electrostatic complex, which is hardly dissolved in the water. Therefore, for the formulation of OPD3 in in vivo oral administration. The in vivo formulation of 5% labrasol and 2.16 mg/kg poloxamer 188 was used[18]. With the formulation, the complex showed clear morphology up to 40mg/ml.

3.3 Characterization of complex

For the characterization of the complex, DSC analysis is performed. DSC analysis shows heat flow of substance, exhibiting exothermal or endothermal tendency over the heating process. As

shown on the Figure 11, docetaxel shows endothermal behavior at 160 to 170 ˚C without any exothermal trend within the observed temperature. DCK shows exothermal behavior around 100 ˚C and exothermal behavior around 160 ˚C. KGDEVD-Docetaxel showed different heat flow tendency from the parent drug docetaxel with showing endothermal behavior at around 100 ˚C and exothermal behavior in around 150 ˚C in broadly spread form. OPD3 complex showed quite broad exothermal and endothermal graph but the peak definitely changed from the KGDEVD-Docetaxel and DCK. This elucidate that the complex with different physical characteristic has been formed.

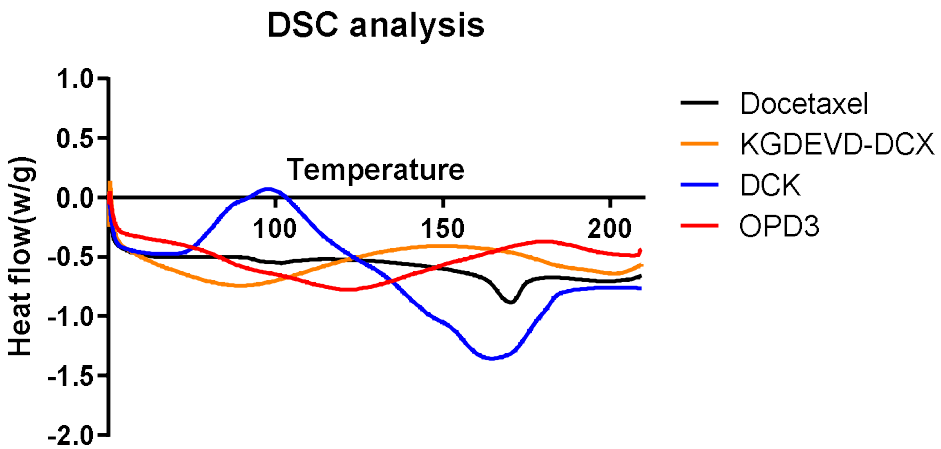

Figure 11. DSC analysis of OPD3

3.4 Cytotoxicity assay

To identify that the triggering targeted therapy Olaparib actually induce apoptosis in target cancer cell, cytotoxicity assay was performed. Since Olaparib only works in BRCA mutant cell lines, BRCA mutant cell line and BRCA wild type cell line from the human pancreatic cancer cell are selected and compared. To control

other genetic effect of the related mechanism in DNA repair system with Olaparib, key gene factor of ATM, ATR, CHEK1, TP53, MDC1 are considered. Cell line which has similar homologous recombinant repair genetic type and different BRCA genotype with Capan-1 is selected; Mia paca-2.

The IC_{50} value of Olaparib was identified in both Capan-1 and Mia paca-2 cell line. In the Capan-1 cell line, IC_{50} value of Olaparib was shown to be 33uM. This value is quite larger than other conventional cytotoxic drugs such as docetaxel or doxorubicin, which has IC_{50} value in nanomolar unit. This is mainly due to the characteristic of Olaparib that works in the DNA repair system. Drugs in the DNA Damage Response (DDR) mechanisms show comparably large IC_{50} value. In contrast to the Capan-1 cell line, Mia paca-2 cell line cannot clarify the IC_{50} value because the cell viability did not fall below 50%. This indicate that Olaparib only works in the BRCA mutant cell line and in the BRCA mutant cell line, it certainly induces apoptosis and cell death.

Moreover, Docetaxel and prodrug DEVD-Docetaxel was also tested for Capan-1 and Mia paca-2 cell line. IC_{50} value of docetaxel was shown to be 228.4 nM in Capan-1 and 93.21 nM in Mia paca-2 respectively. For the docetaxel, Mia paca-2 cell line was more sensitive to the drug then the Capan-1 cell line. Furthermore, this results were comparable to the prodrug form of DEVD-Docetaxel, which exhibited IC_{50} of 863.5 nM in Capan-1 and 575 nM in Mia paca-2 respectively. Prodrug form of docetaxel showed 4 to 6-fold increase in IC_{50} value, elucidating that the sensitivity against the drug has been decreased. Therefore, it is indicated that DEVD-Docetaxel prodrug form showed relatively less toxicity than the parental drug docetaxel.

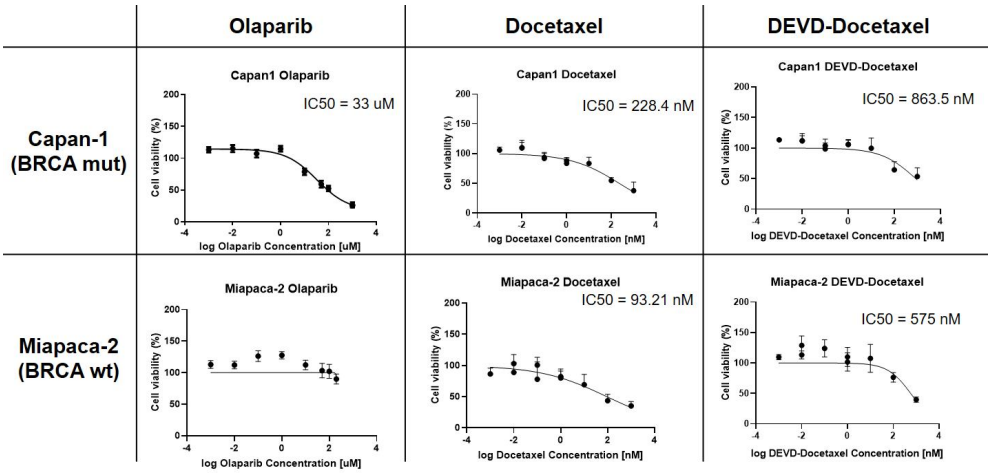

Figure 12. Cytotoxicity assay on Capan-1 and Miapaca-2 cell line with Olaparib, Docetaxel, and DEVD-Docetaxel

3.5 Caspase activity assay

Anti-cancer drug can be classified to the cytotoxic drug and cytostatic drug. Cytotoxic drugs literally attack and kills cancer cells and induce apoptosis of cells. On the contrary, cytostatic drugs render cancer cells to fall into the cell senescence. Cell senescence is different from apoptosis, in that it is more like stop of cell function, until the repair system for the error normally works. Therefore, cytostatic drugs cannot induce apoptosis and release caspse-3, which is crucial in the DEVD peptide strategy.

To clarify if the Olaparib release caspase-3 so that DEVD peptide can be cleaved, Olaparib was treated in different concentrations, and release of caspase-3 was observed. Release of caspase-3 was determined in the fold-increase compared to the control sample. In Figure 13, all Olaparib treated group released caspase-3. The release of the caspase-3 was increased when the treated Olaparib concentration increases. According to the results, Olaparib could induce apoptosis and release caspse-3 in the target cell line Capan-1. Considering that the IC_{50} value of the Olaparib on Capan-1 was 33uM, even lower concentration than IC_{50} also

induced release of caspase-3. This indicates the possibility of reduction of Olaparib dose at the initial induction of apoptosis.

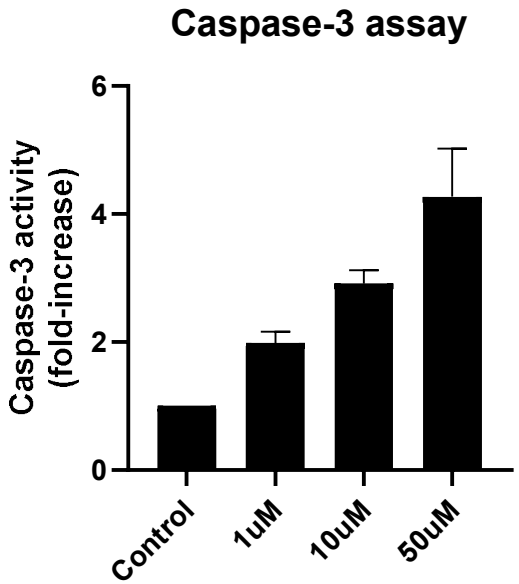

Figure 13. Caspase-3 assay in Capan-1 cell line with Olaparib

3.6 Caspase-3 mediated Docetaxel release

OPD3 is electrostatic complex that is composed of DCK and KGDEVD-Docetaxel. To identify the complex ratio and docetaxel release from OPD3, caspase-3 mediated docetaxel release experiment was constructed.

Caspase-3 mediated docetaxel release was identified by HPLC and LC/MS. Caspase-3 treatment condition was optimized by HPLC. 2, 4 hours after treatment of caspase-3 to OPD3, the sample was analyzed by HPLC. The docetaxel peak area showed no difference in 2 hour time point and 4 hour time point. Therefore, the optimal caspase-3 treatment time was determined to be 2 hours. To quantify the released docetaxel and determine complex ratio, docetaxel standard calibration curve was set. Caspase-3 2 hours treated OPD3 sample was analyzed and docetaxel concentration was

calculated. From the calculated docetaxel concentration, the complex ratio was determined, which was 2.96 : 1 for DCK : KGDEVD-Docetaxel.

To assess reproducibility of data, same experiment was analyzed by LC/MS. Caspase-3 was treated 2 hours and sample was analyzed. From the calculated docetaxel concentration, the complex ratio was calculated to be 3.09 : 1 for DCK : KGDEVD-Docetaxel. Therefore, it was clear that the complex ratio for the OPD3 was 3 : 1.

3.7 Determination of apparent permeability in Caco-2 cells

Caco-2 cells were used to determine apparent permeability of Docetaxel, KGDEVD-Docetaxel, and OPD3. From the transwell system, each substance transported from apical to basolateral side was sampled and analyzed by LC/MS. From the concentrations of each sample, accumulative transported substances graph was derived (Figure 14). To calculate the apparent permeability, the transport system must reach steady-state, which was reached at 2.5 to 3.5 hour time point. In the steady-state, the transported substance amount over time make the steepest slope. From the accumulative transported substance amount, we could already estimate that OPD3 had dramatic increase in apparent permeability.

Docetaxel showed apparent permeability of 0.094 x 10^6 cm/sec, KGDEVD-Docetaxel showed apparent permeability of 0.031 x 10^6 cm/sec. Comparably, OPD3 showed apparent permeability of 0.92 x 10^6 cm/sec, which was around 10-fold larger than that of Docetaxel.

Decrease of apparent permeability in the KGDEVD-Docetaxel group might be due to increase in molecular weight and charge of DEVD peptide which inhibit the paracellular transport of substance. Oral drugs mostly show apparent permeability of around 1 x 10^6 cm/sec to 10 x 10^6 cm/sec. OPD3 showed almost 1 x 10^6 cm/sec of apparent permeability, indicating that there are possibility of oral

absorption. The oral absorption is not totally determined by apparent permeability, so oral absorption must be identified by pharmacokinetic studies.

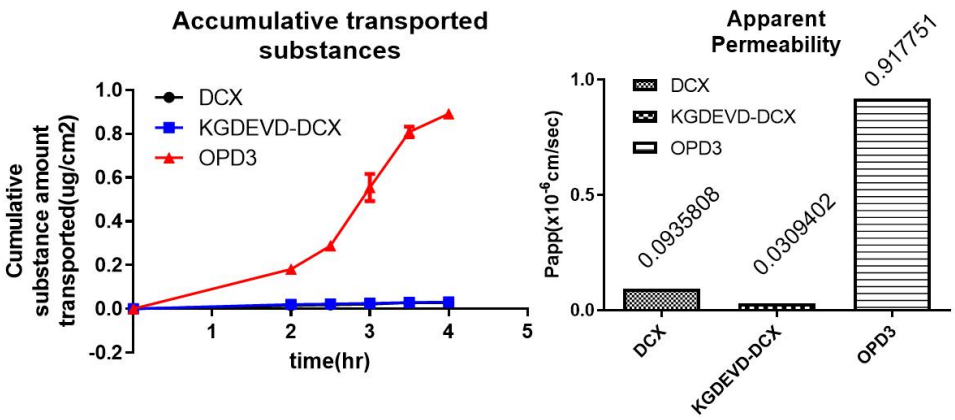

Figure 14. Permeability enhancement evaluation in Caco-2 cells with Docetaxel, KGDEVD-Docetaxel, OPD3

3.8 Absorption mechanism study in Caco-2 cells

To study absorption mechanism of OPD3, the variety of absorption mechanism inhibitors were treated. Actinomycin D was bile acid transport inhibitor. Chlorpromazine was clathrin-mediated endocytosis inhibitor. Brefeldin A was ER/Golgi pathway inhibitor. Geinstein was caveola-mediated pathway inhibitor. Amiloride was macropinocytosis inhibitor. Cyclosporin A was P-gp inhibitor. Since the docetaxel was substrate of P-gp, to assess the effect of OPD3 structure to the P-gp OPD3, OPD3 with cyclosporin A, Docetaxel, Docetaxel with cyclosporin A group was also compared (Table 4).

Among the 6 absorption mechanism inhibitors treated group, Actinomycin D, and chlorpromazine treated group showed significant decrease of apparent permeability. This elucidate that bile acid transport system and clathrin-mediated endocytosis plays

important role in the absorption of OPD3. By complexing DCK, OPD3 binds with ASBT, which is apical sodium bile acid transporter. Also rather than transporting through the ASBT transporter itself, the OPD3 complex is internalized through clathrin-mediated endocytosis and then absorbed through intestine (Figure 15).

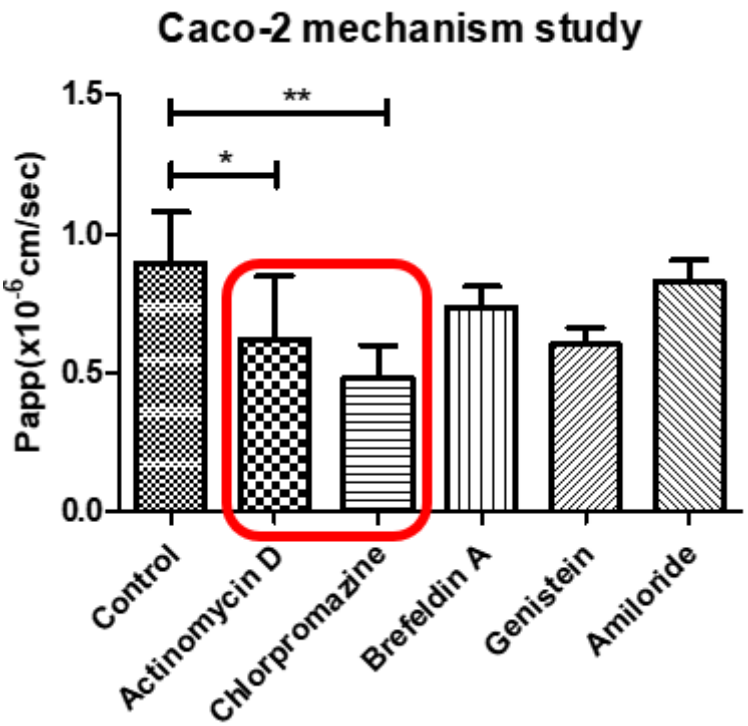

Figure 15. Absorption mechanism study in Caco-2 cells

No.	Inhibitors		Absorption mechanism	Papp (x10^-6 cm/sec)
1	-		Control group(OPD3)	0.9383
2	**Actinomycin D**	**3.2 μM**	**Bile acid transport**	**0.5743**
3	**Chlorpromazine**	**32 μM**	**Clathrin-mediated endocytosis**	**0.4789**
4	Brefeldin A	90 μM	ER/Golgi pathway	0.7351
5	Geinstein	0.1 mM	Caveola-mediated pathway	0.6030
6	Amiloride	0.1 mM	Macropinocytosis	0.8399
7	Cyclosporine A	10 uM	P-gp inhibitor	1.2744
8	Docetaxel			0.09358
9	Docetaxel + Cyclosporin A	10 uM	P-gp inhibitor	0.8688

Table 4. Absorption mechanism study in Caco-2 cells

In the study of effect on P-gp, docetaxel showed around 10 folds increase in apparent permeability when treated with cyclosporin A. However, OPD3 showed about 1.5 folds increase in apparent permeability when treated with cyclosporin A. This indicates that Docetaxel is substrate of P-gp, and released out by P-gp efflux mechanism. Thus, the absorption of docetaxel is indeed enhanced when cyclosporin A is treated. But there was huge variation when treated with cyclosporin A. Also, there was enhancement of bioavailability of docetaxel by cyclosporin A, but there was great variance, showing discontinuity. In contrast, OPD3 showed smaller increase of apparent permeability when treated with cyclosporin A. Therefore, OPD3 structure somewhat provides avoidance to the P-gp efflux mechanism.

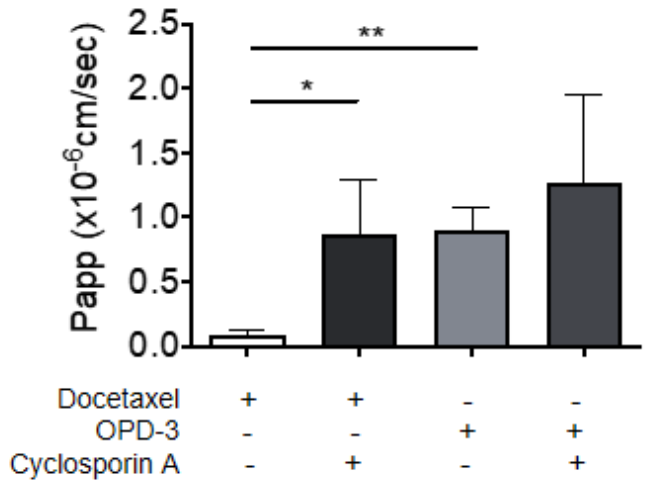

Figure 16. P-gp effect study in the Caco-2 cells

3.9 Pharmacokinetic study

In pharmacokinetic study, docetaxel intravenous and oral group was compared and OPD3 intravenous and oral group was compared. For the oral groups, the dose was set to be 20 mg/kg since low dose administration might cause blood concentration lower than the limit of detection of LC/MSMS. To neutralize the gastric acid, sodium bicarbonate was administered before administration of oral drugs.

Compared to the docetaxel intravenous group, OPD3 intravenous group showed faster decrease of blood concentration. This can be due to faster elimination or larger protein binding factor. Oral docetaxel administration group showed very small blood concentration compared to any groups (Figure 17). Blood docetaxel concentration profile was analyzed by phoenix winnonlin program to obtain pharmacokinetic parameters. According to the analysis, docetaxel oral administration showed bioavailability of 0.43%. Mean residual time for intravenous docetaxel was 1.87 hour and 3.79 for oral docetaxel group. The lagged mean residual time for oral group is due to slow absorption compared to the intravenous administration route. OPD3 oral administration group showed

bioavailability of 32.81%. This can be explained in two aspects. First, the intravenous OPD3 group AUC was decreased copmared to the intravenous docetaxel group. Moreover, oral absorption through bile acid circulation system worked and absorption enhancement happened. Mean residual time of intravenous OPD3 was 0.76 hour, consistent with the result of fast decrease of blood docetaxel concentration in intravenous OPD3 group. Mean residual time of oral OPD3 was 1.85 hour (Table 5).

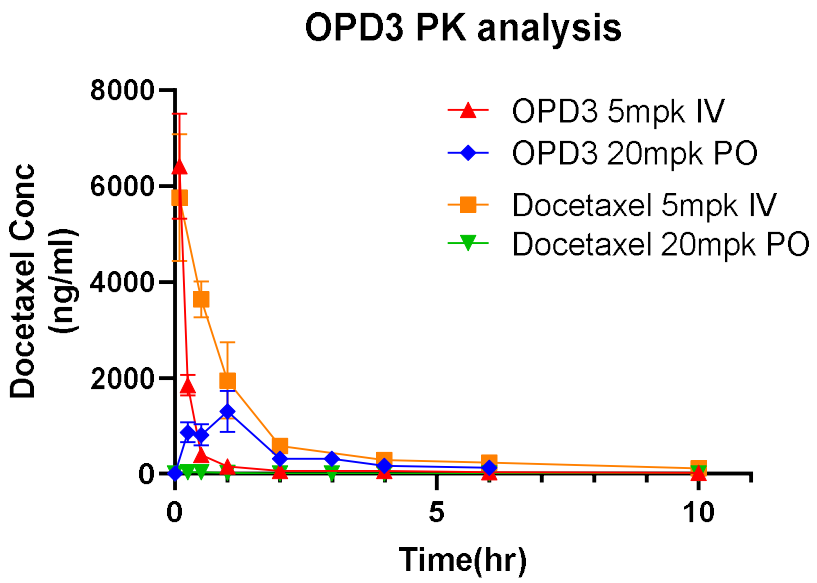

Figure 17. Pharmacokinetic study of OPD3

	Docetaxel		OPD3	
Administration	Intravenous	per oral	Intravenous	per oral
Dose (mg/kg)	5	20	5	20
T_{max} (hr)	0.0833333	0.5	0.0833333	1
C_{max} (ng/ml)	4970.34 ±1101	43.85 ±14.97	5282.05 ±1377	1471.45 ±354
AUC_{last} (ng·hr/ml)	6721.85 ±845	99.25 ±13.8	2427.96 ±276.8	2888.53 ±824
AUC_{Inf} (ng·hr/ml)	7794.62 ±1168	134.61 ±14.54	2466.18 ±272.7	3236.72 ±746.1
MRT_{last} (hr)	1.87 ±0.34	3.79 ±0.3	0.76 ±0.19	1.85 ±0.13
BA (%)	-	0.43±0.05	-	32.81±7.6

Table 5. Pharmacokinetic parameters of OPD3

3.10 Tumor growth suppression study

Tumor growth suppression study was constructed in two different cell lines. First group is Capan-1 xenograft model which is expected to sensitive to OPD3 and Olaparib combination therapy, since Capan-1 is BRCA mutant cell line. According to the 28 days of monitoring, OPD3 5mg/kg treated group showed almost no difference from control group. This is mainly due to the prodrug concept of OPD3, which cannot be activated by triggering agent, Olaparib. Olaparib treated group showed intermediate tumor growth suppression effect. Since Capan-1 is BRCA mutant cell line, Olaparib had effect on inhibiting tumor growth in some degree. Combination of Olaparib and OPD3 5mg/kg or 10mg/kg group showed similar tumor suppression effect and greater effect than Olaparib only treated group. Since the two different dose combination group showed no difference in tumor growth inhibition, there is no need to select 10mg/kg of OPD3 in combination therapy. Combination therapy showed greatest tumor growth inhibition, since activated DEVD peptide can more efficiently kill cancer cell with the apoptosis triggering of Olaparib (Figure 18).

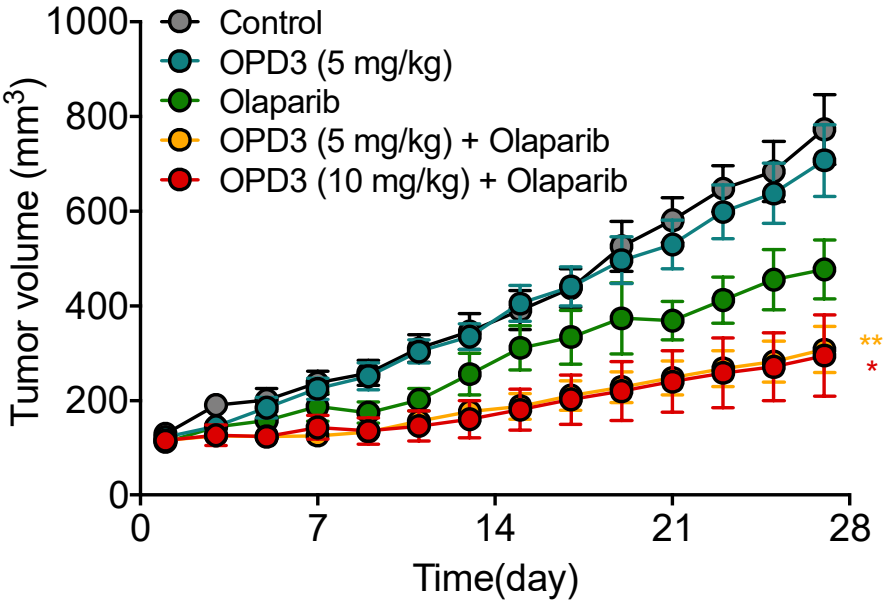

Figure 18. Capan-1 xenograft model tumor volume graph

Tumor weight also showed similar tendency with tumor volume, showing greatest effect in both combination groups. In the aspect of toxicity, body weight was monitored. In the OPD3 10mg/kg group there was body weight decrease observed throughout the monitoring period. The body weight has been decreased 10% from initial weight. This explains the toxicity of combination in high dose. Therefore, the appropriate combination dose regimen should be OPD3 5mg/kg and Olaparib 50mg/kg (Figure 19).

The tumor growth inhibition effect can also be identified in the tumor picture. After 28 days of monitoring, the tumors were sacrificied and the picture was taken (Figure 20).

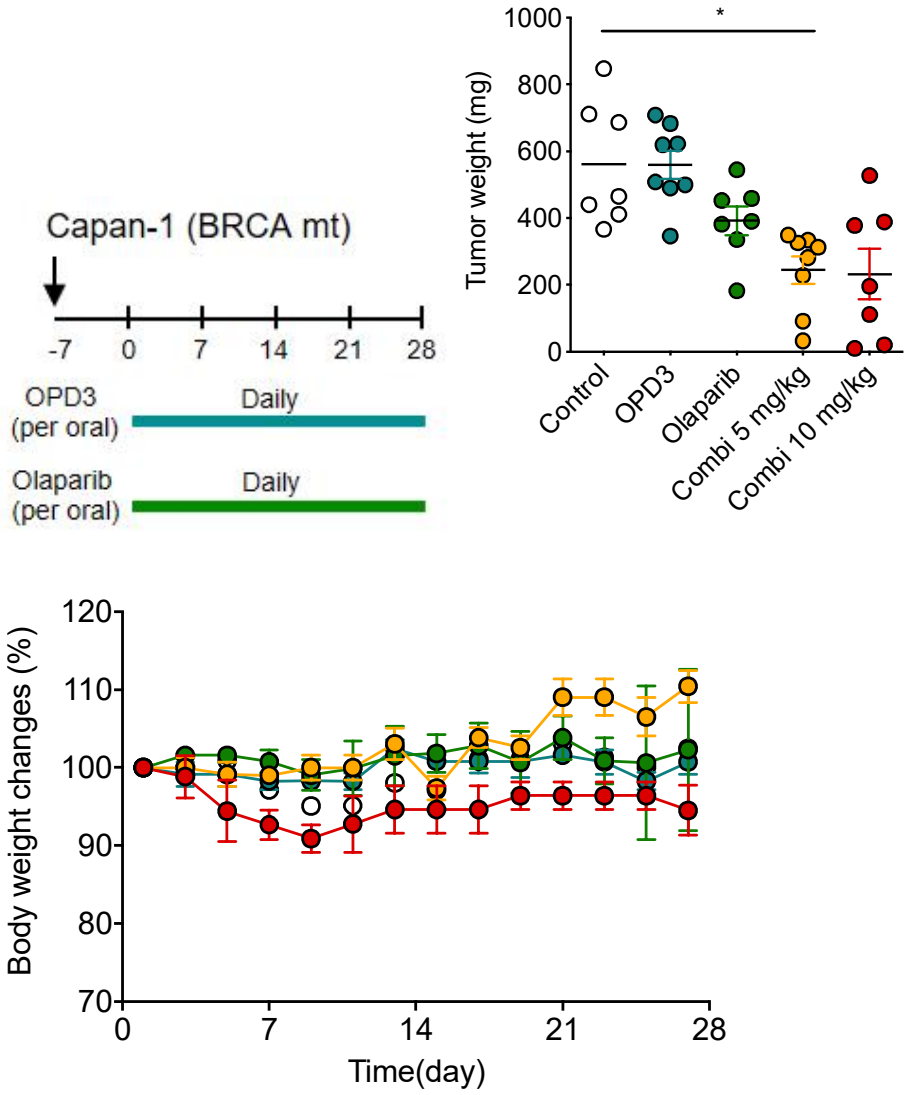

Figure 19. Capan-1 xenograft model dosing schedule, tumor weight, body weight change

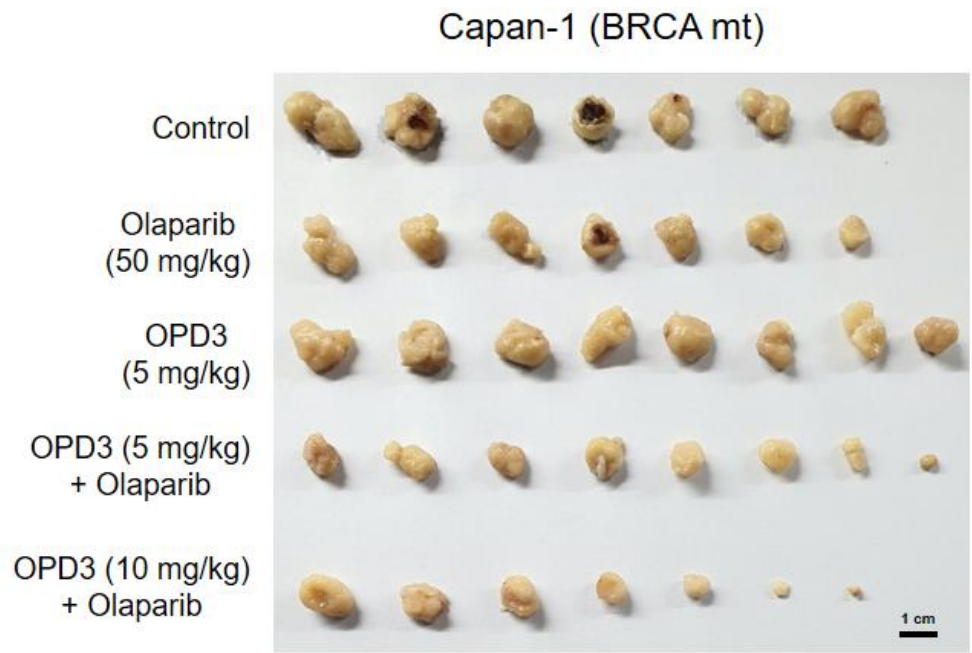

Figure 20. Capan-1 xenograft model tumor picture after sacrifice

Tumor growth inhibition was calculated by following equation.

Tumor growth inhibition (%) =

(Median tumor volume of control group –Median tumor volume of test group) / (Median tumor volume of control group)

OPD3 monotherapy group showed smallest tumor growth inhibition of 5.29 %. Olaparib monotherapy group showed tumor growth inhibition of 34.78%, but it was smaller than combination groups. OPD3 5mg/kg and Olaparib 50mg/kg combination group showed 50.48% of tumor growth inhibition and OPD3 10mg/kg and Olaparib 50mg/kg combination group showed 55.24% of tumor growth inhibition (Table 6).

Group	Tumor Growth Inhibition(%)
Olaparib 50mpk	34.78
OPD3 5mpk	5.29
Combination with OPD3 5mpk	50.48
Combination with OPD3 10mpk	55.24

Table 6. Tumor growth inhibition percent in each groups in Capan-1 xenograft model

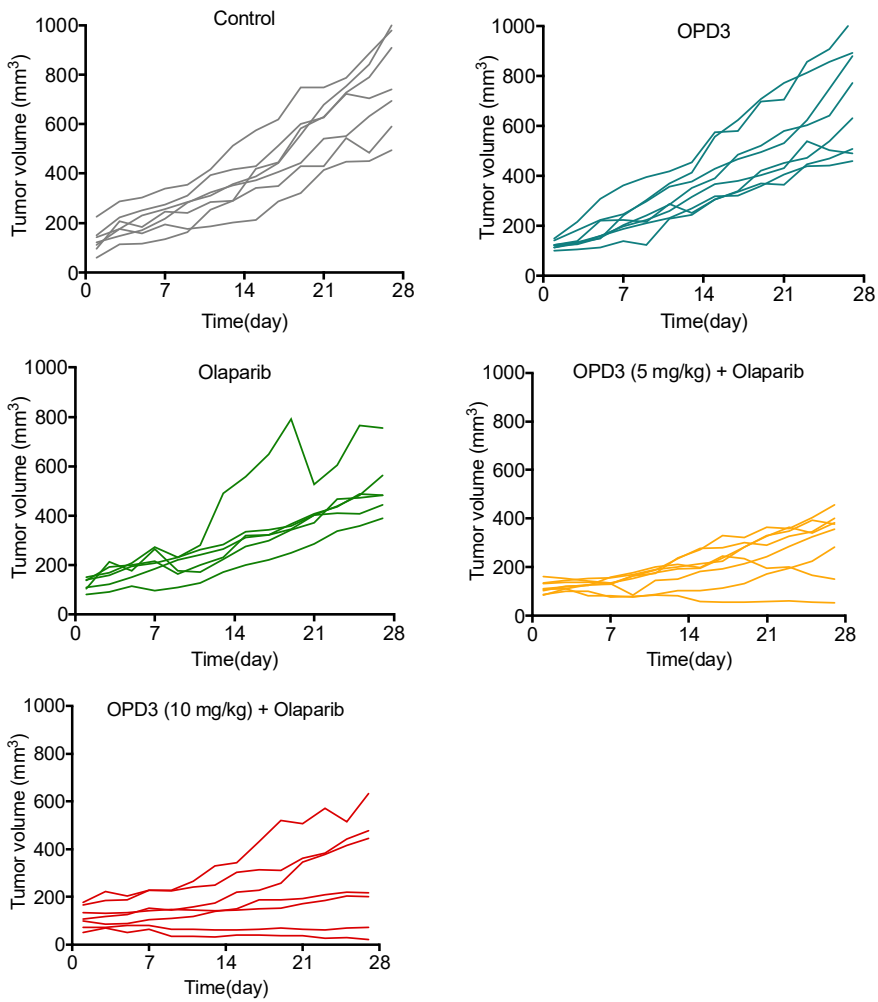

Figure 21. Individual tumor volume change graph of Capan-1 xenograft model

Mia paca-2 cell line is BRCA wild type cell line, which is expected to have no or less effect in combination of Olaparib and OPD3, because Olaparib cannot trigger apoptosis in Mia paca-2. Any group showed significant tumor suppression effect on Mia paca-2 cell line xenograft model. Olaparib cannot induce apoptosis in Mia paca-2 cell line, thus Olaparib monotherapy group and combination group showed almost no effect over the monitoring period (Figure 22).

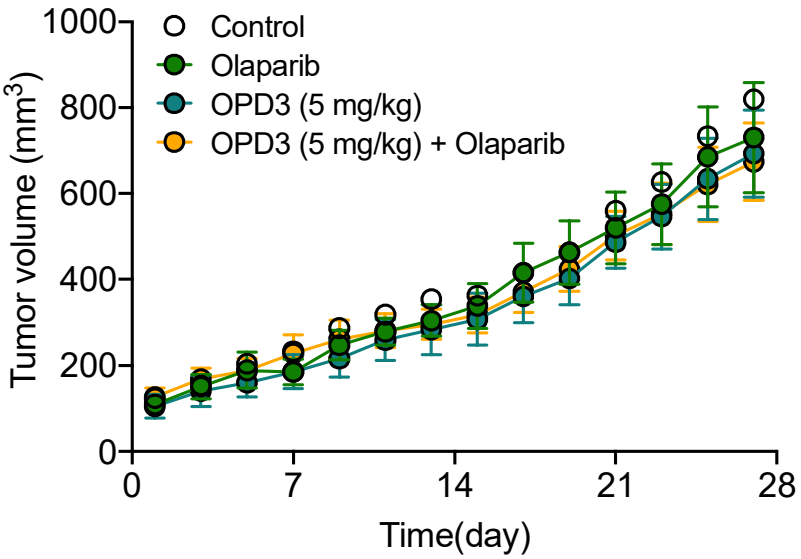

Figure 22. Mia paca-2 xenograft model tumor volume graph

Similar tendency with tumor volume was observed in the tumor weight graph. Since the OPD3 10mg/kg group was excluded by previous toxic profile, there was no body weight loss over 4 groups of study; control, OPD3, Olaparib and OPD3 5mg/kg and Olaparib combination therapy (Figure 23).

Tumor picture also supported the no tumor growth inhibiting effect of either Olaparib, OPD3, and combination group (Figure 24).

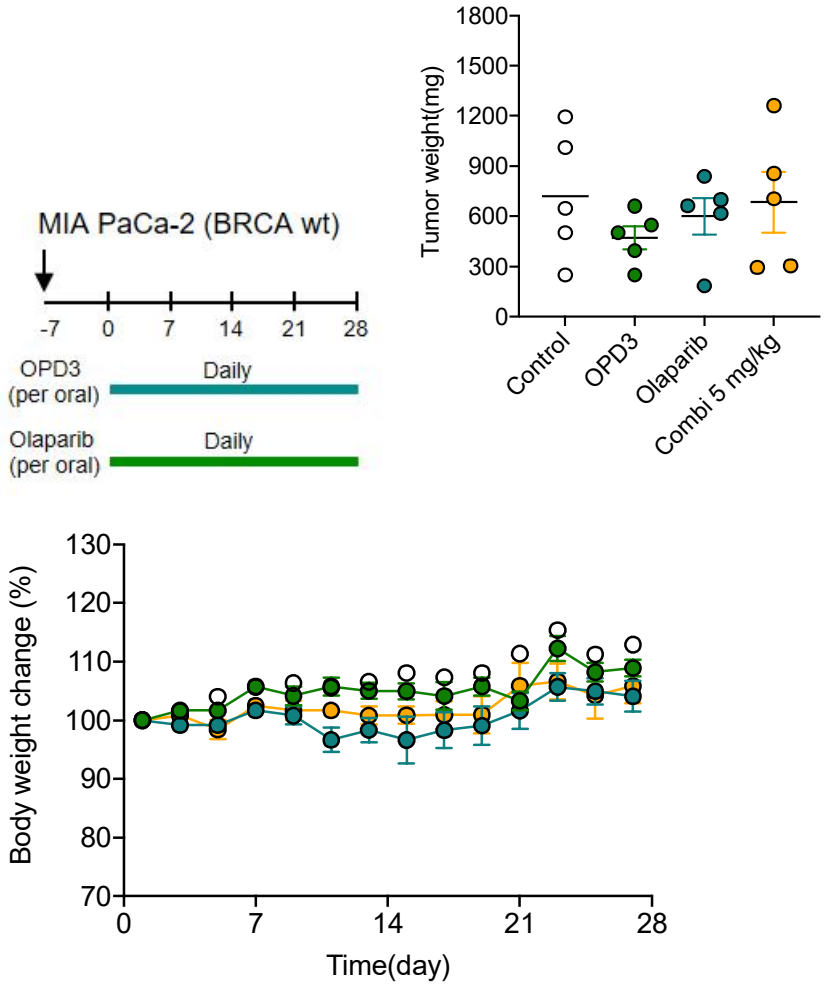

Figure 23. Mia paca-2 xenograft model dosing schedule, tumor weight, body weight change

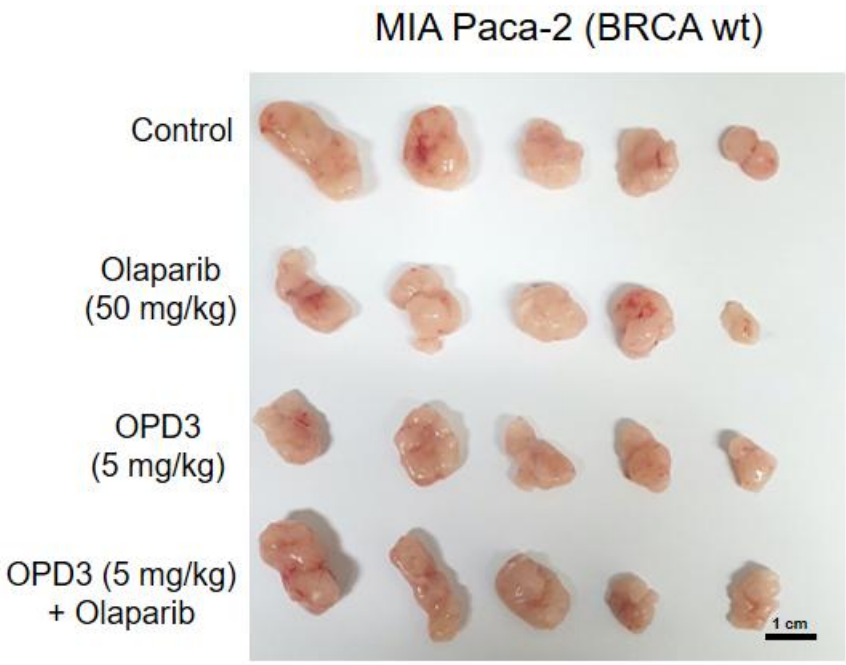

Figure 24. Mia paca-2 xenograft model tumor picture after sacrifice

Tumor growth inhibition rate was also calculated with the same method as used in the Capan-1 xenograft model. Olaparib monotherapy group showed tumor growth inhibition of 11.22%. OPD3 monotherapy group exhibited tumor growth inhibition of 8.75%. Combination group showed tumor growth inhibition of 18.88%. Even if the combination group showed larger tumor growth inhibition, it was not showing any synergystic effect. Rather, the value was even smaller than the additive value of the each therapy group. This elucidate that there was no synergy effect of combination therapy, and Olaparib did not work at the Mia paca-2 xenograft model (Table 7).

Group	Tumor Growth Inhibition(%)
Olaparib 50mpk	11.22
OPD3 5mpk	8.75
Combination with OPD3 5mpk	18.88

Table 7. Tumor growth inhibition percent in each groups in Mia paca-2 xenograft model

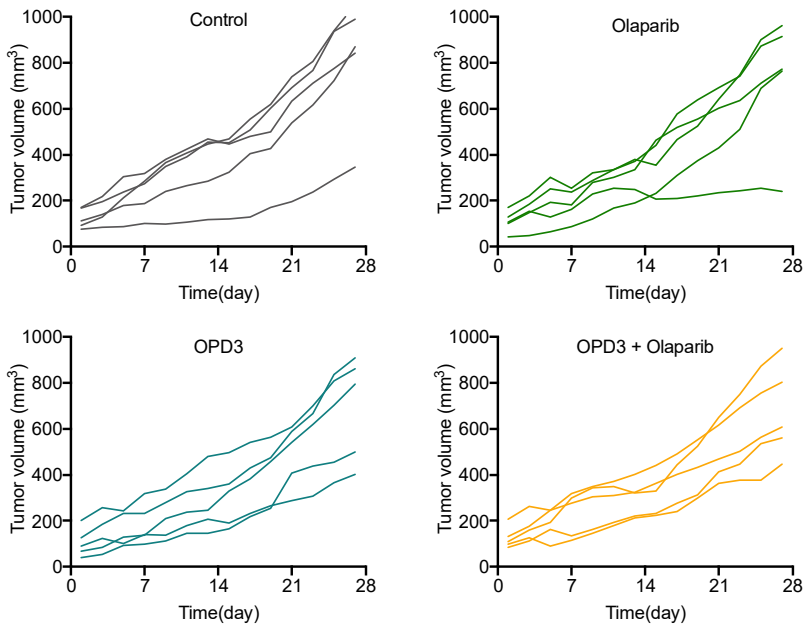

Figure 25. Individual tumor volume change graph of Mia paca-2 xenograft model

3.11 Liver metastasis model in Capan-1 pancreatic cancer cell line

To establish liver metastasis model, the surgery techniques were first tested. Methylene blue dye was injected directly through portal vein to clarify that the injection through portal vein deliver cells to the liver directly (Figure 26A).

After confirming the surgical techniques, 4T1 murine breast cancer cell line was tested for the liver metastasis model. 4T1 murine breast cancer cell was selected because it grows very rapidly, thus it is easier to identify the growth of tumor on liver and determine the success or failure of surgery. 4T1 murine breast cancer cell injected mice reported death on Day 12, and survived mice were sacrificed. There was multiple metastatic nodule on the liver and also out grown tumor tissue attached on the liver. Therefore, it was successful in murine liver metastasis model (Figure 26B, C).

The next trial was to move on to the human cancer cells. The final goal of the project was applying OPD3 and Olaparib combination on BRCA mutant metastatic cancer cells. Therefore, BRCA mutant human cancer cell candidate Capan-1 pancreatic cancer cell and HCT-116 colorectal cancer cell was selected. In human cancer cell liver metastasis model, the nodules were smaller than that of the murine liver metastasis model. HCT-116 human colorectal cancer cell model showed more aggressive growth of nodule or tumor tissue over the liver (Figure 26D, E, F). Capan-1 human pancreatic cancer cell model showed less nodules grown on the liver, and even no nodules were found on the liver (Figure 26G, H, I).

There are some reasons that led to unsuccessful results in human cancer cell liver metastasis model. First, cancer cells were less aggressive than the murine cancer cells. Therefore, more cells were needed for single injection. Also murine cells were allogenic so that there was no immune resistance. In the contrary, human cancer cells were xenogeneic, which is immunogenic. Even if the

mice were selected as balb/c nu mice, which have less immune system, it is difficult for human cancer cells to thrive. Moreover, the single injection cell number is limited, because the injection volume is limited. Too large volume injected through liver can damage liver and infarct other capillary vessels. Furthermore, portal vein injection method took 15 or more minutes per one mouse surgery. This was unproductive to secure large amount of experimental samples.

Therefore, the second surgery method of intra-splenic surgery was suggested. Intra-splenic surgery has advantage in more simple surgical method and shorter time required for single surgery. Intra-splenic surgery method is now taken and research over such method is going on. In addition to the change of the surgical method, the luciferase transfected cell line, Capan-1-luc2, has been introduced. Luciferase transfected cells allow the non-invasive monitoring of the surgery progression by bioluminescence. Further study will be conducted on this luciferase transfected cell and anti-tumor effect on liver metastasis will be evaluated.

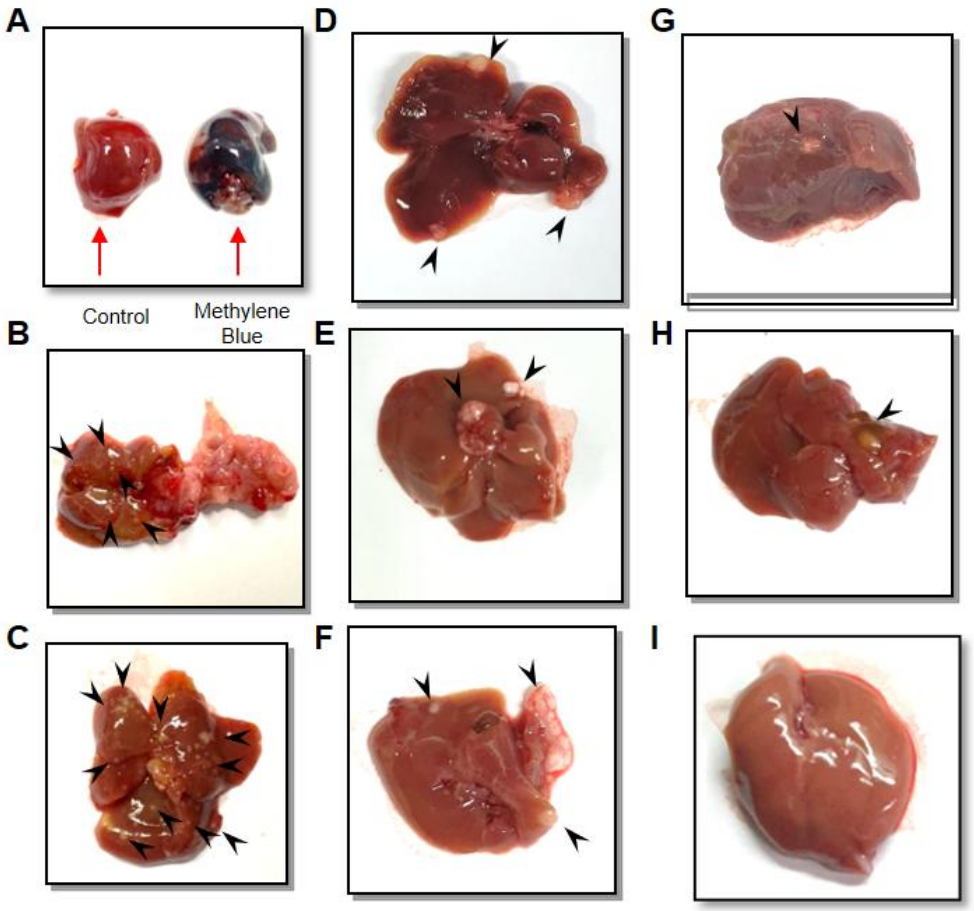

Figure 26. Liver metastasis model establishment. A, comparison of control liver to liver injected with methylene blue through portal vein. B, C, 4T1 liver metastasis model on day 12. D, E, F, HCT-116 liver metastasis model on day 23, 30, 42 respectively. G, H, I, Capan-1 liver metastasis model on day 32, 42, 63 respectively.

Chapter 4. Conclusion

Drug resistance is the old problem in the anti-cancer field. The main factor causing drug resistance is tumor heterogeneity. Tumor heterogeneity is morphologic, genetic, and phenotypic diverse in the inter or intra tumor. Typical drug resistance come from genomic instability in intratumor heterogeneity. Even the primary tumor was sensitive to the specific drug, repeated treatment of drug induce drug resistance subclones to thrive and drug resistance new subclones arise by genomic evolution. This procedure is called clonal selection, the process of tumor to obtain drug resistance. If altered resistant tumor metastasis, the metastatic tumor cannot be treated with the initial drug.

DEVD peptide prodrug system is suggested to overcome such resistance hurdle. In the contrary to the typical targeted agents that targets tumor physiological targets such as receptors or ligands, DEVD peptide prodrug strategy rather target biological event, "apoptosis". By exploiting the characteristic of caspase-3 protein, which is released from the apoptotic event, that it specifically recognizes and cleaves DEVD peptide, DEVD conjugated drug can be activated with the exposure to the caspase-3. Activated drug can further affect adjacent cancer cells and induce secondary apoptosis and caspase-3 release. This recurrent chain reaction brings activation of prodrug at the cancer site. Former researches utilized radiation therapy to induce initial apoptosis at local tumor. In this study, targeted therapy was used to induce primary apoptosis at metastatic tumor, since radiation therapy cannot be used at all metastatic lesions.

Metastatic cancers can be treated in the metronomic maintenance regimen. This differ from conventional maximum tolerable dose schedule, in that frequent and low dose is administered. MMC prefers oral drugs for frequent dosing, so this research exploited the bile acid derivative to orally deliver DEVD

peptide prodrug. Among the bile acid derivatives, deoxycholic acid and lysine conjugate (DCK) is selected. It formed complex with DEVD peptide prodrug by electrostatic hydrophobic force.

For the triggering targeted therapy, Olaparib was selected. It is PARP inhibitor, and recently approved for the metastatic pancreatic cancer. Olaparib targets BRCA mutant cancer cells and induce apoptosis.

In this study, DEVD docetaxel peptide conjugate prodrug and DCK is complexed to synthesize OPD3. This drug was used with Olaparib. Whether the Olaparib induce apoptosis and release caspase-3 is identified by in vitro experiments. Pharmacokinetic study was conducted to assess pharmacokinetic profile of OPD3. Through absorption mechanism study it is revealed that OPD3 is absorbed via ASBT and clathrin mediated endocytosis. By comparing Capan-1 and Mia paca-2 xenograft model, it is confirmed that OPD3 and Olaparib combination therapy had effect on only BRCA mutant cell line Capan-1. Further study on liver metastasis model is ongoing.

In sum, this study devise new material of OPD3 in the treatment of metastatic pancreatic cancer with the use of Olaparib as triggering agent. The genotype specific anti-tumor effect on xenograft model has been confirmed and evaluation over liver metastasis model is under process.

Metastatic pancreatic cancer is treated with limited drug spectrum. Therefore, optimizing drug payload on prodrug should be done on further study. Although liver metastasis model on murine cancer cells were successful, human cancer cell liver metastasis model is incomplete. There should be development in establishing human cancer liver metastasis model before evaluating combination effect.

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국문초록

약물 저항성은 항암 약물의 치료에 있어서 큰 장벽이다. 이러한 약물 저항성의 기저 원인은 종양 이질성에 있다. 특히, 종양내 이질성은 약물 처리가 반복됨에 따라 저항성 소집단 선택성을 보여 지속적 항암 치료에 걸림돌이 되어왔다.

종양 이질성에 의한 약물 저항성을 회피하기 위해 본 논문에서는 DEVD 펩타이드 약물 접합체를 제시한다. DEVD 전구약물은 유도 형질 표적 치료 전략을 사용하는데, 이는 세포자멸을 표적으로 하여 세포자멸 과정에서 방출된 Caspase-3가 DEVD를 특이적으로 인지하고 절단한다는 점을 이용하여 세포자멸에 의해 전구약물의 활성화를 일으킨다. 이러한 전략을 전이성 췌장암에 적용하기 위해 표적치료제 올라파립과의 병용을 고안하였다.

또한, 경구 메트로노믹 유지 요법에 근거하여 전이암을 치료하기 위해 DEVD 전구약물을 경구화하였다. 담즙산 유래 물질의 복합체가 경구 흡수를 촉진한다는 점을 이용하여 DCK를 이용하여 OPD3 복합체를 합성하였다. 본 연구를 통해 OPD3 의 약물 동태학적 파라미터와 흡수기전을 규명하였고, 올라파립과 병용요법으로써 췌장암 마우스 이종 이식 모델에서 종양 억제능을 평가하였다. 올라파립은 BRCA 유전자 돌연변이 환자군에서 특이적으로 사용할 수 있기 때문에 BRCA 돌연변이 전이성 췌장암에 대한 약효를 평가하였다. 추가로 전이성 췌장암의 가장 높은 표적 장기인 간 전이 모델의 확립을 시도하였다. 궁극적으로 약물 저항성을 회피하고 전신독성이 적은 BRCA 유전자 돌연변이성 간 전이성 췌장암에 대한 OPD3와 올라파립의 병용 요법의 가능성을 시사하였다.

주요어: 종양 이질성, DEVD, 전이성 췌장암, 메트로노믹 유지요법, 담즙산 유래 물질, 올라파립

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