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Ph.D. Dissertation

# Characterization of SN38-resistant T47D breast cancer cell sublines overexpressing BCRP, MRP1, MRP2, MRP3, and MRP4

Graduate School of Chosun University

Department of Medicine

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SN38 내성 T47D 유방암 아세포주의 특성

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# Characterization of SN38-resistant T47D breast cancer cell sublines overexpressing BCRP, MRP1, MRP2, MRP3, and MRP4

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## BCRP와 MRP1, MRP2, MRP3, MRP4를 과발현하는 SN38 내성 T47D 유방암 아세포주의 특성

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**배경** 유방암은 여성에서 흔한 악성 암으로 매년 발생율이 증가하고 있다. 대부분의 유방암은 항암치료를 필요로 하며, 다른 고형암에 비해서 비교적 반응이 좋다. 그러나 항암제에 대한 다약제내성(multidrug resistance, MDR)으로 인해 치료 실패가 발생하게 된다. 이를 극복하기 위해서 항암제 내성 유전자와 이러한 내성 유전자를 발현하는 내성 세포주를 개발하기 위한 연구가 진행되고 있다. 그러나 유방암 내성단백질(breast cancer resistance protein, BCRP)을 발현하는 세포주는 많지 않다. Camptothecin 유도 topoisomerase I 억제제에 내성을 보이는 암세포주에서 BCRP의 과발현함이 보고되었고 이는 irinotecan과 SN38 7-ethyl-10-hydroxycamptothecin)에 교차내성(cross-resistance)을 보였다. 이에 본 연구에서는 Irinotecan의 활성 대사물질인 SN38을 이용하여 BCRP를 발현하는 새로운 유방암 내성 세포주를 확립하고, 내성기전을 규명하고자 하였다.

**방법** T47D 유방암 야생형 세포(wild type T47D, T47D/WT)에 SN38의 농도를 단계적으로 증가시켜 SN38 내성 T47D 유방암 아세포주를 확립하였다. 약제 감수성은 methyl thiazolyl tetrazolium (MTT) 방법으로 측정하였고, 세포형태 변화는 현미경을 통해 관찰하였다. 내성 아세포주에서 항암제 내성진단유전자 검출키트(Drugsporter®), 실시간 역전사-중합효소 연쇄반응(real time RT-PCR)과 Western blot 분석으로 다약제내성 유전자 발현을 분석하였다. 내성 세포내의 약물 농도는

유세포 분석법 (flow cytometry)을 이용하여 측정하였다.

**결과** 유방암 세포주 T47D를 SN38 120 nM과 150 nM에 장기간 노출시켜 SN38 내성 T47D 유방암 아세포주인 T47D/SN120과 T47D/SN150를 얻었다. T47D/SN120과 T47D/SN150 세포는 T47D/WT 세포에 비해서 SN38에 각각 14.5배 및 59.1배의 내성을 보였고, 두 세포주는 mitoxantrone(각각 3.4배 및 26.4배)과 topotecan(각각 4.9배 및 12.0배)에 교차내성을 보였다. 이러한 내성 세포는 BCRP와 다약제내성단백질 1, 2, 3 및 4 (MRP1, MRP2, MRP3, MRP4)를 과발현하였다. 또한, DNA methyltransferase 억제제인 5-azadeoxycystidine과 histone deacetylase 억제제인 trichostatin A를 T47D/WT 세포에 투여하였을 때 MRP1, MRP2, MRP3, MRP4 및 BCRP mRNA 발현이 증가하였다. 두 내성 세포주의 세포내 약물 농도는 야생형 세포에 비해서 낮았으나, MRP 억제제인 probenecid와 BCRP억제제인 genistein 투여는 두 수송체의 형광기질약물의 세포내 농도를 증가시켰을 뿐 아니라 항암제의 감수성도 증가시켰다.

**결론** T47D/SN120과 T47D/SN150 세포는 DNA methylation과 histone deacetylation에 의해 발생하는 후생학적 침묵(epigenetic silencing)이 억제됨으로써 BCRP와 MRP1, MRP2, MRP3, MRP4를 과발현하여 MDR 표현형을 보였으며 이는 이미 알려진 화학감작제(chemosensitizers) 투여로 역전 되었다. 이와 같이 다양한 내성 단백질을 발현하는 SN38 내성 T47D 유방암 아세포주는 다약제 내성 기전의 규명과 함께 새로운 화학감작제 개발을 위해 유용하게 사용될 것으로 사료된다.

Key words: 유방암; SN38; 다약제내성단백질 (multidrug resistance protein, MRP); 유방암내성단백질 (breast cancer resistance protein, BCRP); 화학감작제 (chemosensitizer)

## I. Introduction

Breast cancer is characterized by a malignant proliferation of epithelial cells of the ducts or lobules of the breast [1]. It is second most common malignancy following thyroid cancer and sixth leading cause of cancer death in Korean women. In 2011, 16,015 persons were newly diagnosed and 2,013 patients died in Korea. Breast cancer is responsible for 7.3% (14.8% in women) of all cancer incidence and 2.7% (7.3% in women) of all cancer deaths. The incidence of breast cancer has gradually increased to 5.9% (6.1% in women) per year [2].

The treatment of breast cancer is largely classified into two groups: local treatment, which includes surgery, radiation, or both modalities, and systemic treatment, which includes cytotoxic chemotherapy, endocrine therapy, biologic therapy, or a combination of these. Systemic treatment is used in the adjuvant, neoadjuvant, and palliative settings. Consequently, systemic treatment plays an important role in the treatment of breast cancer at various stages [3]. Indeed, systemic agents are effective at the beginning of therapy in 85-95% of primary breast cancers [4-8] and in 50% of metastatic cancers [9-11].

However, after a variable duration of chemotherapy, cancer progression often occurs owing to the development of resistance to chemotherapy. Most cases of metastatic breast cancer present an acquired resistance to treatment [12].

Chemotherapy resistance is divided into primary resistance and acquired resistance. Primary resistance corresponds to cases where a response is not observed despite the use of the appropriate initial chemotherapy and the tumor continues to grow during the treatment [13]. Acquired resistance corresponds to cases where tumor cells seem to respond well to the initial chemotherapy, but they acquire resistance to the anticancer drugs due to repeated exposure [14]. In this case, cells are resistant to some agents of the same class, but are sensitive to drugs of different classes. However,

eventual cross-resistance to multiple anticancer drugs of apparently different structures and functions is observed. This phenomenon is known as multidrug resistance (MDR) [15].

The mechanisms and pathways of MDR are complicated and multifactorial. One of the mechanisms of MDR is associated with alteration of anticancer drug transporter. Among them, a classic MDR mechanism is associated with decreased drug accumulation by increasing the drug efflux pump in the tumor cell membrane [16]. This MDR phenotype is mediated by ATP (adenosine triphosphate)-binding cassette (ABC) transporters [17], including P-glycoprotein [18,19] and multidrug resistance protein (MRP) family members [15,20,21]. Another novel transporter, breast cancer resistance protein (BCRP), was identified as an ABC half-transporter and distributed in the placenta and various cancer types [22-25]. Meanwhile, it was reported that a redistribution of the anticancer drug from the nucleus to the cytoplasm is related to MDR as non-ABC transporters [26]. Lung resistance protein (LRP) is involved in the nucleo-cytoplasmic transport and cytoplasmic sequestration of anticancer drugs [27-29]. Another mechanism of MDR is associated with alterations in topoisomerase [30,31].

Despite a comprehensive knowledge about mechanisms of drug resistance, the nature of chemotherapy resistance in breast cancer and the potential role of drug resistant genes, involved in the transport or sequestration of anticancer agents, are still unclear [32]. Moreover, exploring the mechanism of MDR and finding molecular targets for drug resistance is important for the development of new treatments. Thus, further studies for a better understanding of MDR mechanisms in breast cancer and for the development of resistant cancer cell lines of various types are vital.

Recently, several novel resistant breast cancer cell lines have been established. Notably, BCRP expression is observed in primary breast cancer as well as in normal breast tissue [33-35], but only few resistant breast cancer cell lines overexpressing BCRP are available [36-39]. Tumor cell lines resistant to camptothecin-derived topoisomerase I inhibitor, topotecan,

overexpressed BCRP and showed a significant cross-resistance to CPT11, SN38 (7-ethyl-10-hydroxycamptothecin), and 9-aminocamptothecin [40-43]. SN38, an active metabolite of irinotecan, possesses a much stronger cytotoxicity against tumor cells than irinotecan [44,45], through inhibition of DNA topoisomerase I [46]. However, so far, no SN38-resistant breast cancer cell sublines have been developed.

Therefore, the specific aim of this study was to establish SN38-resistant breast cancer cell sublines, anticipating the overexpression of transporters, such as BCRP. Herein, two SN38 resistant breast cancer cell lines, T47D/SN120 and T47D/SN150, characterized by MRP1, MRP2, MRP3, MRP4 and BCRP overexpression are described. These resistant cancer cell sublines will be used to develop chemosensitizers that can reverse the resistance.

## II. Materials and Methods

### 1. Cell culture

The human breast cancer cell line T47D was purchased from the Cancer Research Center in Seoul National University (Seoul, South Korea). The cells were cultured in RPMI-1640 (GibcoBRL Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Sigma Chemical Co. St. Louis, MO, USA). The cells were allowed to adhere to the culture dish and to form a monolayer. They were subcultured once they reached confluence.

### 2. Establishment of the SN38-resistant breast cancer cell sublines

The SN38-resistant breast cancer cell sublines, T47D/SN120 and T47D/SN150 were established by gradually increasing the concentration of SN38 from 15 nM ($IC_{50}$ value) to final concentrations of 120 nM ($IC_{50}$ x 8) and 150 nM ($IC_{50}$ x 10), respectively. Stable T47D/SN120 and T47D/SN150 cell lines were obtained in 16 months.

### 3. Calculation of cellular population doubling time

The cells were seeded into 24-well culture plates at 5 x $10^4$ cells per well, and incubated at 37 ℃ for 24 h. The cell numbers were counted every 24 h for 4 days. The cell doubling time (Td) was calculated using the formula: $Td = T \times \log 2/(\log N_t - \log N_0)$, where $N_0$ and $N_t$ represent the number of cells at the beginning and at the end of the culture during time T, respectively.

### 4. Chemosensitivity test using MTT assay

The methyl thiazolyl tetrazolium (MTT; Sigma Chemical Co. St. Louis, MO, USA) assay was performed to assess sensitivity of the T47D/SN120 and

T47D/SN150 to anticancer drugs. The 50% inhibitory concentration ($IC_{50}$) was defined as the drug concentration that causes a 50% reduction in the number of cells compared with the untreated control. The $IC_{50}$ values were determined directly from the dose-response curves. Resistance factor (RF) was calculated by the ratio of the $IC_{50}$ values of T47D/SN120 and T47D/SN150 to T47D/WT cells.

## 5. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) assay

Total RNA was extracted from the cells using the RNeasy mini kit (Qiagen, Hilden, Germany). mRNA expression was determined by RT-PCR and was normalized to *β-actin* mRNA expression level. Genes as well as the conventional and real-time primer pairs are listed in Table 1.

The RNAs were reverse transcribed with units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) and the oligo (dT) primer for 1 h at 37 ℃. The synthesized cDNA was diluted 1:5 with water and was amplified with 2.5 units of Taq polymerase (TaKaRa, Tokyo, Japan) and 10 *p*mol of each primer, under each PCR defined conditions, using a GeneAmp PCR9600 (Perkin-Elmer-Cetus, Waltham, MA, USA). After the final cycle, all the PCR products were subjected to a final extension for 5 min at 72 ℃. The PCR products were electrophoresed. The endpoint used in PCR quantification (Ct) was defined as the PCR cycle number that crosses an arbitrarily placed signal threshold.

## 6. Western blot analysis

The cells were washed with phosphate-buffered saline (PBS) and lysed in 50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA). The cell lysates were

centrifuged and then fractionated by sodium dodecyl sulfate-polyacrylamide gell electrophoresis (SDS-PAGE). Western blotting was performed using a slight modification of the method described previously [47]. The membrane was incubated with primary rabbit polyclonal antibodies for MRP1 (1:1000; Crpinc), MRP2 (1:5000; Sigma), MRP3 (1:50; Abcam, Cambridge, England), MRP4 (1:50; Abcam), BCRP (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (1:2500; Santa Cruz Biotechnology). The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:2500 for BCRP and β-actin) for 1 h. The signal was then detected using the ECL detection kit (Amersham, Piscataway, NJ, USA).

## 7. Drug accumulation assay

Cell suspensions ($5 \times 10^5$ cells) in PBS were exposed to 1 μM rhodamine 123, 50 nM calcein-AM, and 20 μM mitoxantrone at 37 °C for 1 h. Additionally, cells were incubated in the presence of each fluorescent substrate with 5 μM PSC833, 5 mM probenecid, 100 μM genistein, and 2 mM cyanide in PBS at 37 °C for 1 h. After incubation, cellular drug accumulation was determined using a flow cytometer (FACSCalibur, Becton Dickinson, MA, USA), which detected drug fluorescence. A focused argon laser beam (488 nm) excited the cells in a laminar sheath flow and fluorescence emissions at 530 nm for rhodamine 123 and calcein-AM, and at 670 nm for mitoxantrone were collected to generate the histogram.

## 8. Screening of chemosensitizers

$IC_{50}$ values of SN38 were obtained in the presence and absence of chemosensitizers for SN38-resistant T47D sublines and the ratios were defined as chemosensitizing index.

## 9. Statistical analysis

Statistical significance of the data was determined using a Student's *t*-test. *P* values less than 0.05 were considered significant.

## III. Results

### 1. Sensitivity of the T47D/WT, T47D/SN120 and T47D/SN150 cells to SN38 and other anticancer drugs

SN38-resistant T47D/SN120 and T47D/SN150 cell sublines were established from the wild-type T47D cells after long-term exposure, more than 16 months, to 120 nM and 150 nM SN38, respectively. Microscopic observation showed some distinct features of SN38-resistant T47D sublines compared to their parenteral cell line. T47D/WT cells were relatively consistent in size and shape in monolayer, while the resistant cells presented a spindle-shaped morphology and their size was smaller than that of T47D/WT cells (Fig 1). Moreover, T47D/SN120 or T47D/SN150 cell population doubling time was shorter than that of T47D/WT (35.5 h vs. 69.2 h and 32.8 h vs. 69.18 h, respectively).

The MTT assay showed that T47D/SN120 and T47D/SN150 cells were more resistant to SN38, irinotecan, and topotecan (14.5 and 59.1 times, 1.5 and 3.7 times, and 4.9 and 12 times, respectively) as compared with the wild-type drug-sensitive parental cells (Fig. 2). In addition to topoisomerase I inhibitors, both T47D/SN120 and T47D/SN150 sublines were cross-resistant to various anticancer drugs that have been used in breast cancer treatment, including microtubule inhibitors (paclitaxel and vinblastine), antimetabolites (5-fluorouracil and methotrexate), topoisomerase II inhibitors (doxorubicin and mitoxantrone), estrogen receptor blockers (tamoxifen and endoxifen), an alkylating agent (cisplatin), and a tyrosine kinase inhibitor (gefitinib) (Table 2).

As shown in Table 2, both T47D/SN120 and T47D/SN150 sublines were highly resistant to microtubule inhibitors, including paclitaxel and vinblastine. On the other hand, the T47D/SN150 subline presented weak resistance to gefitinib, 5-FU, and irinotecan, and was sensitive to methotrexate and endoxifen as compared with the T47D/SN120 subline.

## 2. Expression profiles of 24 transporters in the T47D/SN sublines

The expression profiles of the resistance-related transporters were examined in T47D/WT, T47D/SN120, and T47D/SN150 cells using the resistance diagnostic kit (Drugsporter®). Drugsporter® is composed of 24 transporter genes and one house keeping gene GAPDH [48]. Among the 24 transporter genes, only MRP2 mRNA increased from about two folds to ten folds in a concentration-independent manner in T47D/SN120 and T47D/SN150 cells treated with 3.5 nM to 90 nM of SN38. After increasing the concentration to 120 nM and 150 nM, a high level of BCRP, MRP1, MRP2, MRP3, and MRP4 mRNA expression was detected in T47D/SN120 and T47D/SN150 cells. There was no difference in mRNA expression of other genes between T47D/WT cells and SN38-resistant T47D sublines. The real-time RT-PCR assay confirms that T47D/SN120 and T47D/SN150 cells overexpressed MRP1 (7-fold and 11-fold, respectively), MRP2 (795-fold and 1,051-fold, respectively), MRP3 (57-fold and 96-fold, respectively), MRP4 (204-fold both), and BCRP (536-fold and 3,083-fold, respectively) compared to T47D/WT cells (Fig. 3).

MRP1, MRP2, MRP3, MRP4 and BCRP protein expression was determined by western blot analysis. Fifty micrograms of protein was used to detect MRP1, MRP2, MRP3, and MRP4. MRP1, MRP2, MRP3, MRP4 expression levels in T47D/SN150 cells were compared with those of T47D/SN120 cells. T47D/SN150 cells overexpressed MRP1 protein (1.2-fold), MRP2 protein (1.1-fold), MRP3 protein (1.1-fold), and MRP4 protein (2.2-fold) compared to T47D/SN120 cells (Fig. 4).

BCRP protein expression in T47D/SN120 and T47D/SN150 cells was increased by 85.3-fold and 327.5-fold, respectively compared with that of T47D/WT cells, which expressed a trace amount of BCRP. T47D/SN150 cells overexpressed BCRP 4-fold compared with T47D/SN120 cells (Fig. 4).

### 3. Sensitivity of SN38-resistant T47D sublines to SN38 in the presence of various chemosensitizers.

The effects of several chemosensitizers including Pgp inhibitors (verapamil, PSC833, and 3,4,5-trimethoxyflavone (TMF)), the MRP inhibitor (probenecid), and the BCRP inhibitor (genistein) was assessed on SN38-resistant T47D sublines. Among the Pgp inhibitors, only TMF showed some chemosensitizing effects on SN38 in a concentration-dependent manner. Probenecid and genistein also sensitized SN38-resistant T47D sublines to SN38 in a concentration-dependent manner (Fig 5). The chemosensitizing effects of TMF and genistein to SN38 were similar between T47D/SN120 and T47D/SN150 cells. As shown in Table 3, the chemosensitizing index was calculated by dividing the $IC_{50}$ value in the absence of chemosensitizer with that in its presence. Chemosensitizing indices of probenecid (5 and 50 μM) and genistein (1 and 10 μM) were 1.3 - 2.0 and 1.2 - 10.8, respectively in both cell lines, whereas verapamil (1 and 10 μM) and PSC833 (5 and 50 nM) were less than 1.0, with the exception of PSC833 in the T47D/SN120 cell line, which presented a chemosensitizing index of 1.3.

### 4. Involvement of epigenetic gene silencing of MRP1, MRP2, MRP3, MRP4, and BCRP in T47D/WT cells

Next, we investigated whether MRP1, MRP2, MRP3, MRP4, and BCRP could be epigenetically induced in T47D/WT cells after treatment with 2.5 μM 5-aza-2'-deoxycytidine (AdC) for 96 h or 100 ng/mL trichostatin A (TSA) for 48 h. RT-PCR indicated that AdC induced mRNA expression of MRP2, MRP3, MRP4, and BCRP, while TSA induced mRNA expression of MRP1, MRP2, MRP4, and BCRP in T47D/WT cells (Fig. 7).

## 5. Decreased drug accumulation in T47D/SN120 and T47D/SN150 cells

The drug accumulation study was performed to estimate the functional activity of transporter in T47D/SN120 and T47D/SN150 cells. Calcein AM and mitoxantrone used as fluorescent substrates for MRP and BCRP, respectively, and detected by flow cytometry. Accumulation of both substrates was decreased in T47D/SN120 and T47D/SN150 cells compared with that of T47D/WT cells. However, treatment with the inhibitors, probenecid and genistein increased the accumulation of both substrates (Fig. 8, Fig. 9).

On the other hand, intracellular levels of rhodamine 123, a substrate of Pgp, were not affected after treatment with PCS833, a Pgp inhibitor in T47D/SN120 and T47D/SN150 cells (Fig 10).

## IV. Discussion

Breast cancer is the second most common cancer in women, and leads to considerable morbidity and mortality. Breast cancer accounts for 20% of female cancer prevalence [2]. Although development of breast cancer prognosis and treatment continuing, chemotherapy resistance still frequently occurs and is a major problem in the management of breast cancer. Eventually, MDR develops in most of the systemic recurrent or initially metastatic breast cancer patients. Therefore, overcoming MDR could improve the efficacy of chemotherapy.

The aim of this study was to discover genes associated with MDR in refractory breast cancer and to establish new resistant breast cancer cell lines in order to develop new chemosensitizers to eventually overcome MDR.

We established two new resistant breast cancer cell sublines, T47D/SN120 and T47D/SN150. Both sublines showed a high resistance to various anticancer drug, including paclitaxel and vinblastine as well as SN38. Additionally, T47D/SN150 cells showed a high resistance to mitoxantrone, topotecan, and doxorubicin. Generally, levels of resistance are classified as high resistance (RF > 20 x), moderate resistance (RF 5-15 x), and low or no resistance (RF < 5 x ) [49].

Compared to their parental cell line, T47D/SN120 and T47D/SN150 cells presented a faster proliferation and a shorter doubling time. These features were not consistent with other resistant cell line such as MCF-7/TAX [50], BEL-7402/5-FU [51], MCF-7/Doc, and MCF-7/Adr [36]. This result may be the result of a short incubation period. Most of the cells grow following a pattern S-shaped growth curve, but we calculated the doubling time at a time prior to the S shape growth curve.

The mRNA expression profiles of the transporter genes in T47D/SN120 and T47D/SN150 cells were compared with that of the T47D/WT cell line. We found that T47D/SN120 and T47D/SN150 cells overexpress MRP1, MRP2, MRP3, MRP4, and BCRP. Moreover, T47D/SN150 cells obtained by exposure

to higher concentration of SN38, expressed a greater amount of transporter genes associated with MDR. In other word, T47D/SN150 cells expressed a greater amount of MDR-related gene and showed a higher resistance to anticancer drugs. This suggests that multidrug resistance is related to the drug concentration used as well as the presence of MDR-related gene.

MTT assays were performed to assess the sensitivity of SN38-resistant T47D sublines to SN38 in the presence of inhibitors (Pgp inhibitor: verapamil, PSC833, and TMF; MRP inhibitor: probenecid; BCRP inhibitor: genistein). TMF, probenecid, and genistein showed a concentration-dependent chemosensitizing effect. Although MRP and BCRP expression in T47D/SN150 cells was higher than that of T47D/SN120 cells, TMF and genistein chemosensitizing effect were similar in T47D/SN120 and T47D/SN150 cell lines. It suggests that the concentrations of TMF and genistein used in this study were enough to occupy all transporter binding sites. Interestingly, administration of the well-known Pgp inhibitors, verapamil and PSC833 did not sensitize the cell to SN38, but the sensitivity was recovered by administration of TMF. In addition, the sensitivity recovery pattern observed in SN38-resistance breast cancer cells treated with TMF was similar to that observed in SN38-resistance breast cancer cells treated with genistein. This suggests that TMF not only functions as a Pgp inhibitor, but also affects other mechanisms, reversing MDR. Further studies are warranted to determine TMF functions.

Next, experiments were designed to identify the relationship between MRP1, MRP2, MRP3, MRP4, and BCRP expression and epigenetic gene silencing. Generally, alteration of gene function in cancer is attributed to either genetic alterations such as mutations or deletions, or epigenetic alterations, which alter gene expression status [52]. Epigenetic alterations include promoter methylation and chromatin remodeling such as histone modification without DNA sequence alteration [53]. The changes of gene expression caused by epigenetic alterations in cancer can be divided into three categories, transcriptional regression by methylation of the promoter CpG island, which is

lesions of the genome rich in sequences of a cytosine preceding a guanine, increased gene expression by hypomethylation, decreased gene expression associated with histone deacetylase (HDAC) [54]. Aberrant DNA methylation in normally unmethylated gene promoter CpG island results in a decrease of gene expression. Hypermethylation of promotor CpG island of tumor suppressor genes is a hallmark of all human cancers [55,56]. Moreover, global hypomethylation of the DNA contributes to carcinogenesis by inducing genomic instability [57-59]. DNA hypomethylation is associated with activation of proto-oncogenes [60]. Last, activated histone deacetylase (HDAC) induces promoter DNA methylation and represses of gene expression [52,61]. Consequently, epigenetic gene silencing occurs by DNA hypermethylation and histone deacetylation. Therefore, DNA methyltransferase inhibitors and histone deacetylase inhibitors, which inhibit DNA methylation and histone deacetylation, induce the expression of genes that were abnormally suppressed and have been used as therapeutic agents to treat cancer caused by epigenetic gene silencing. In this study, expression of MRP 2, MRP3, MRP4, and BCRP was significantly increased in T47D/WT cancer cells treated with the DNA methyltransferase inhibitor, 5-azadeoxycystidine (5-Adc). Similarly, histone deacetylase inhibitor, trichostatin A, treatment of T47D/WT cancer cells significantly increased expression of MRP1, MRP2, and MRP4, and BCRP. In other words, contrary to what occurs in cancer induced by epigenetic gene silencing, overexpression of MRP1, MRP2, MRP3, MRP4, and BCRP is caused by DNA demethylation and histone acetylation. These results suggest that expression of MRP1, MRP2, MRP3, MRP4, and BCRP is induced by the suppression of epigenetic gene silencing. These findings are consistent with reports indicating that epigenetic gene silencing is involved in irinotecan sensitivity in colorectal cancer cells [62-64]. Intracellular irinotecan is inactivated by UGT1A1 or pumped out by transporters such as ABCB1 (Pgp), ABCC1 (MRP1) and ABCC2 (MRP2) [65, 66]. Since silencing of UGT1A1 and transporters occurs by DNA methylation, DNA methyltransferase inhibitors can restore gene expression and thereby enhances SN38

inactivation [63]. ABC transporter gene silencing occurs by histone deacetylation [62, 64]. In this study, surprisingly, expression of MRP3 decreased after treatment with TSA. The mechanism that leads to such a result still remains to be elucidated, but is difficult to explain because studies regarding epigenetic profiling of the ABC loci are still incomplete.

BCRP, MRP1, MRP2, MRP3, and MRP4 decreased intracellular drug accumulation. When the BCRP and MRP inhibitors, genistein and probenecid were added as chemosensitizers, intracellular drug concentrations were increased. However, the addition of the Pgp inhibitor, PSC833, did not increase intracellular drug accumulation. This suggests that T47D/SN120 and T47D/SN150 cell resistance to anticancer drugs is not associated with Pgp. Additionally, these inhibitors presented a synergic effect with ATP depletion induced by cyanide. As previously demonstrated by others, we were also able to confirm that MRP1, MRP2, MRP3, MRP4, and BCRP are ATP-dependent transporters.

In summary, two SN38-resistant breast cancer cell sublines, T47D/SN120 and T47D/SN150, were established by gradual exposure to SN38. These resistant cells overexpressed MRP1, MRP2, MRP3, MRP4, and BCRP in a SN38 concentration-dependent manner. These resistant cells present a higher resistance than their parental wild type breast cancer cells to various anticancer drugs. Intracellular substrates of anticancer agents were decreased in both SN38-resistant breast cancer cell sublines, but were increased by known chemosensitizers. T47D/SN120 and T47D/SN150 cells were chemosensitized to SN38 by TMF, probenecid, and genistein. In this study, new SN38-resistant breast cancer cell sublines overexpressing MRP1, MRP2, MRP3, MRP4, and BCRP were established. These cancer cell lines can be useful for the development of new chemosensitizers.

## V. Conclusion

T47D/SN120 and T47D/SN150 cells overexpressed MRP1, MRP2, MRP3, MRP4, and BCRP by the suppression of epigenetic gene silencing, which occurs by DNA hypermethylation and histone deacetylation. These resistant cells presented the classical MDR phenotype characterized by cross resistance to various other anticancer drugs. This MDR phenotype decreased intracellular drug accumulation, but it was reversed by known chemosensitizers such as probenecid and genistein. Thus, these SN38-resistant T47D breast cancer cell sublines can be useful for the screening of novel chemosensitizers.

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Table 1. Primer sequences of PCR

| Gene | Primer | Conventional | Real-time |
|---|---|---|---|
| BCRP | Sense | 5' CAGGTCTGTTGGTCAATCTCAC 3' | GCCTACAACTGGCTTAGACT |
| | Antisense | 5' CAGTGTGATGGCAAGGGAAC 3' | GATGATTGTTCGTCCCTGCT |
| MRP1 | Sense | 5' CTAACCTGGACCTGGAACTG 3' | GGTCAGCCCAACTCTCTTG |
| | Antisense | 5' TCAATCAACACTGTAAGCAACC 3' | ACTGAACTCCCTTCCTCCTC |
| MRP2 | Sense | 5' AACCTCATTCAGACGACCATCC 3' | TCAGGTTTGCCAGTTATCCG |
| | Antisense | 5' GACCATTACCTTGTCACTGTCC 3' | TGGTTGGTGTCAATCCTCAC |
| MRP3 | Sense | 5' CTCCAAGACAGAGACAGAGGC 3' | CCTGCTACTTGCTCTACCTG |
| | Antisense | 5' TGGCCCACGCTGAGATTCTC 3' | ACACCCAGGACCATCTTGA |
| MRP4 | Sense | 5' AACCTCTAACCGACATTCCTG 3' | GGGAGAGAACCAGCACTTC |
| | Antisense | 5' TCAACATATTACAGCCACCATC 3' | TGCTGTTTCCAAGGCATCT |

Table 2. The sensitivity of the T47D/WT, T47D/SN120 and T47D/SN150 cells to SN38 and other anticancer drugs

| Drug | T47D/WT | T47D/SN120 | T47D/SN150 |
|---|---|---|---|
| | $IC_{50}$ (RF) | | |
| Paclitaxel | 0.007 μg/mL ±0.00039 (1) | 15.54 μg/mL ±3.380 (2,220) | 41.45 μg/mL ±1.519 (5,921) |
| Vinblastine | 0.017 μg/mL ±0.00035 (1) | 33.04 μg/mL ±1.115 (1,943) | 39.96 μg/mL ±3.099 (2,350) |
| Doxorubicin | 0.056 μg/mL ±0.0095 (1) | 0.585 μg/mL ±0.082 (10.4) | 1.07 μg/mL ±0.079 (19.1) |
| SN38 | 0.017 μg/mL ±0.0037 (1) | 0.247 μg/mL ±0.014 (14.5) | 1.004 μg/mL ±0.200 (59.1) |
| Topotecan | 0.16 μg/mL ±0.036 (1) | 0.79 μg/mL ±0.229 (4.9) | 1.92 μg/mL ±0.179 (12.0) |
| Gefitinib | 23.53 μg/mL ±0.745 (1) | 97.11 μg/mL ±2.910 (4.1) | 97.21 μg/mL ±2.274 (4.1) |
| Mitoxantrone | 0.057 μg/mL ±0.032 (1) | 0.196 μg/mL ±0.036 (3.4) | 1.51 μg/mL ±0.224 (26.4) |
| Endoxifen | 25.83 μg/mL ±4.318 (1) | 79.34 μg/mL ±0.172 (3.1) | 68.05 μg/mL ±2.091 (2.6) |
| 5-FU | 2.89 μg/mL ±0.214 (1) | 8.19 μg/mL ±0.773 (2.8) | 9.83 μg/mL ±0.389 (3.4) |
| Irinotecan | 10.24 μg/mL ±0.399 (1) | 14.98 μg/mL ±0.357 (1.5) | 38.03 μg/mL ±2.372 (3.7) |
| Cisplatin | 118.84 μg/mL ±27.13 (1) | 159.51 μg/mL ±7.822 (1.3) | 194.17 μg/mL ±8.421 (1.6) |
| Tamoxifen | 46.06 μg/mL ±3.512 (1) | 59.65 μg/mL ±2.437 (1.3) | 66.64 μg/mL ±2.750 (1.4) |
| Methotrexate | 53.91 μg/mL ±6.316 (1) | 57.72 μg/mL ±2.379 (1.1) | 43.48 μg/mL ±8.457 (0.8) |

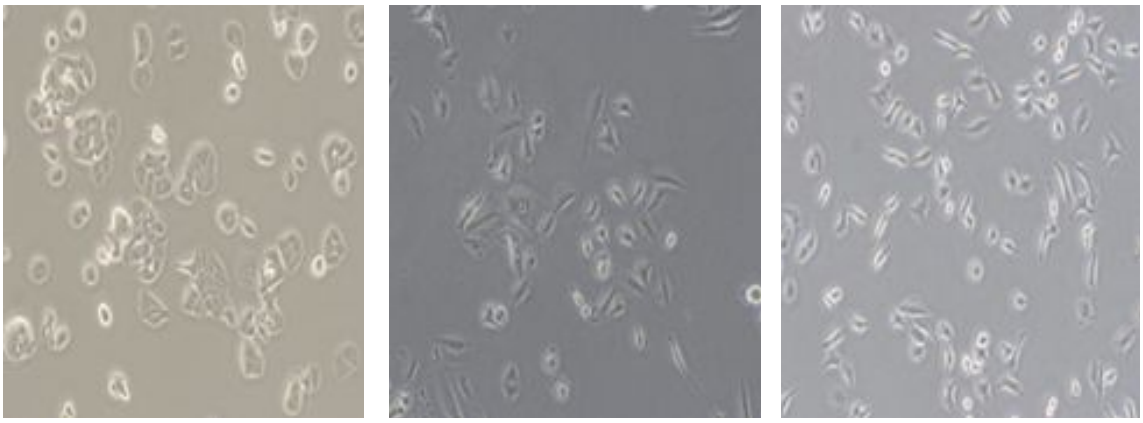
Drugs are sorted according to the order of relative resistance of T47D/SN120 cells to T47D/WT cells.

Table 3. Effect of chemosensitizers on SN38-resistant T47D sublines

| | Chemosenensitizing index (%) of chemosensitizers | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | verapamil (μM) | | PSC833 (nM) | | TMF (μM) | | Probenecid (μM) | | Genistein (μM) | |
| | 1 | 10 | 5 | 50 | 5 | 50 | 5 | 50 | 1 | 10 |
| SN120 | 0.56 | 0.88 | 0.67 | 1.30 | 3.01 | 7.73 | 1.31 | 1.72 | 1.28 | 3.90 |
| SN150 | 0.48 | 0.50 | 0.65 | 0.60 | 15.95 | 10.56 | 2.00 | 1.99 | 7.53 | 10.78 |

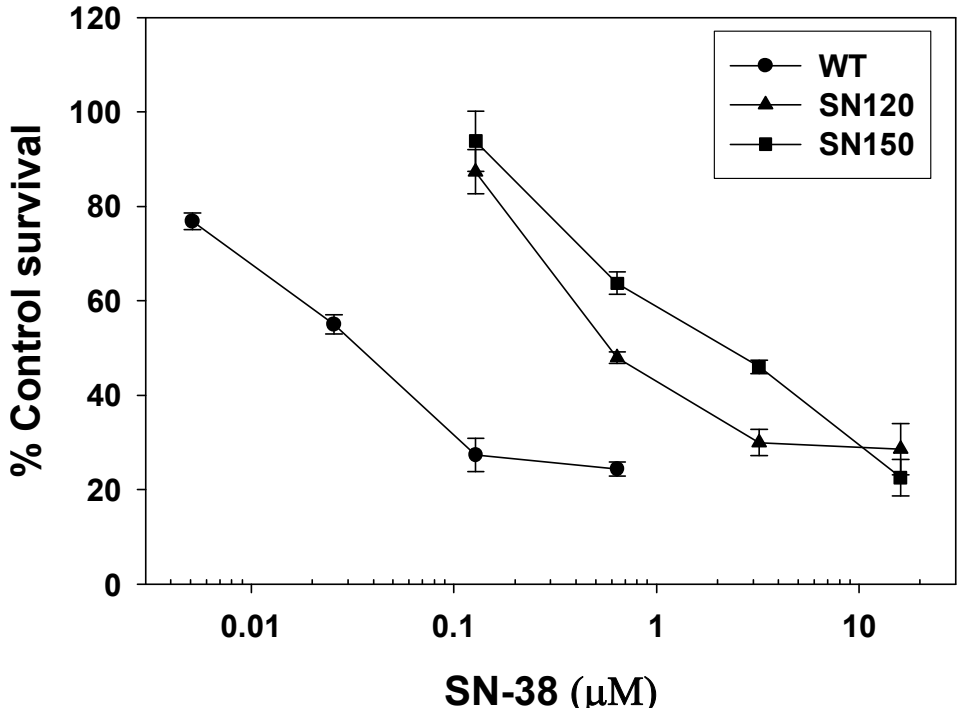
Chemosensitizing index (%) =

$$\frac{IC_{50} \text{ of SN38 in the absence of chemosensitizer}}{IC_{50} \text{ of SN38 in the presence of chemosensitizer}}$$



T47D/WT T47D/SN120 T47D/SN150

Figure 1. Morphological change in SN38-resistant T47D sublines.



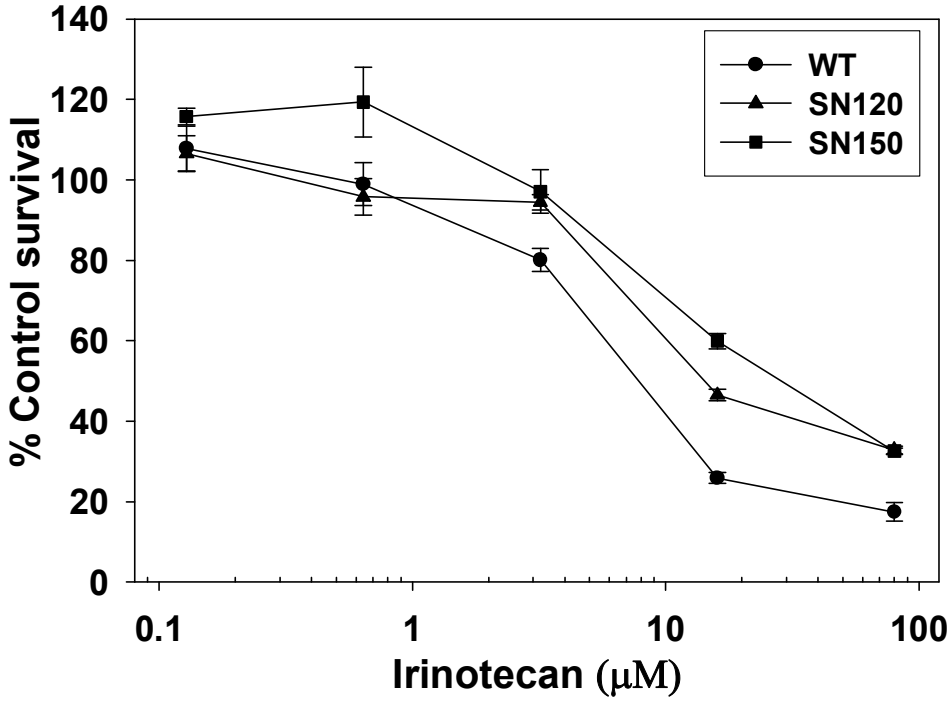




Figure 2. Sensitivity of the T47D/WT, T47D/SN120 and T47D/SN150 cells to SN38 and irinotecan. Sensitivity was determined by the MTT assay.

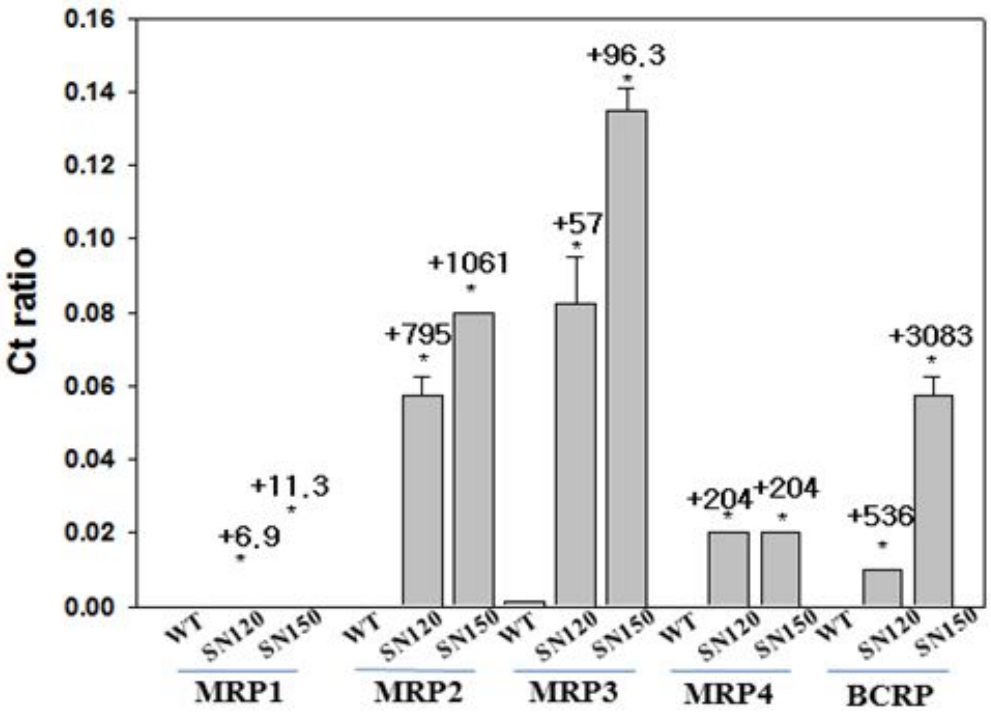
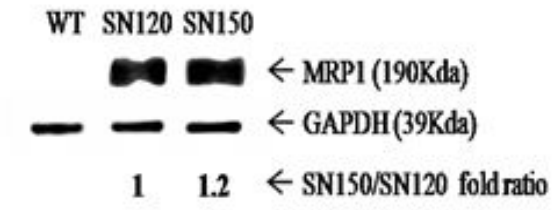
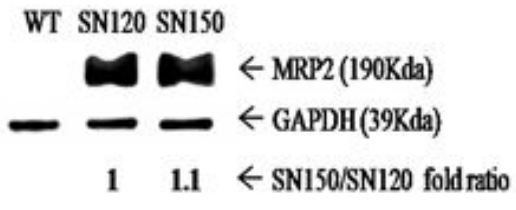



Figure 3. Real-time RT-PCR assay for MRP1, MRP2, MRP3, MRP4 and BCRP mRNA expression of the T-47D/WT, T-47D/SN120 and T-47D/SN150 cells. Numbers above column refer relative fold increase as compared with WT cells.
*, $P < 0.05$ vs WT

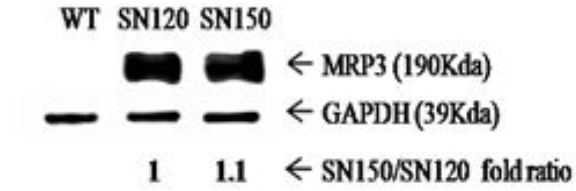
1) MRP1



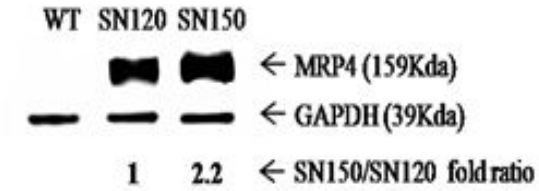

2) MRP2




3) MRP3




4) MRP4




5) BCRP

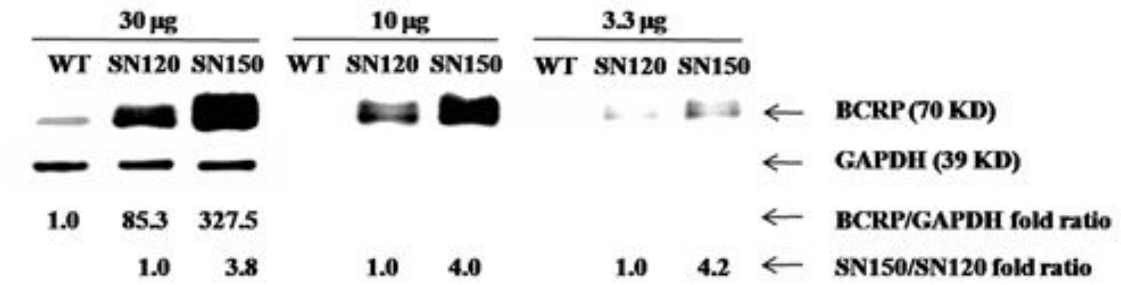



Figure 4. Western blot analysis for MRP1, MRP2, MRP3, MRP4 and BCRP protein of the T47D/WT, T47D/SN120 and T47D/SN150 cells.

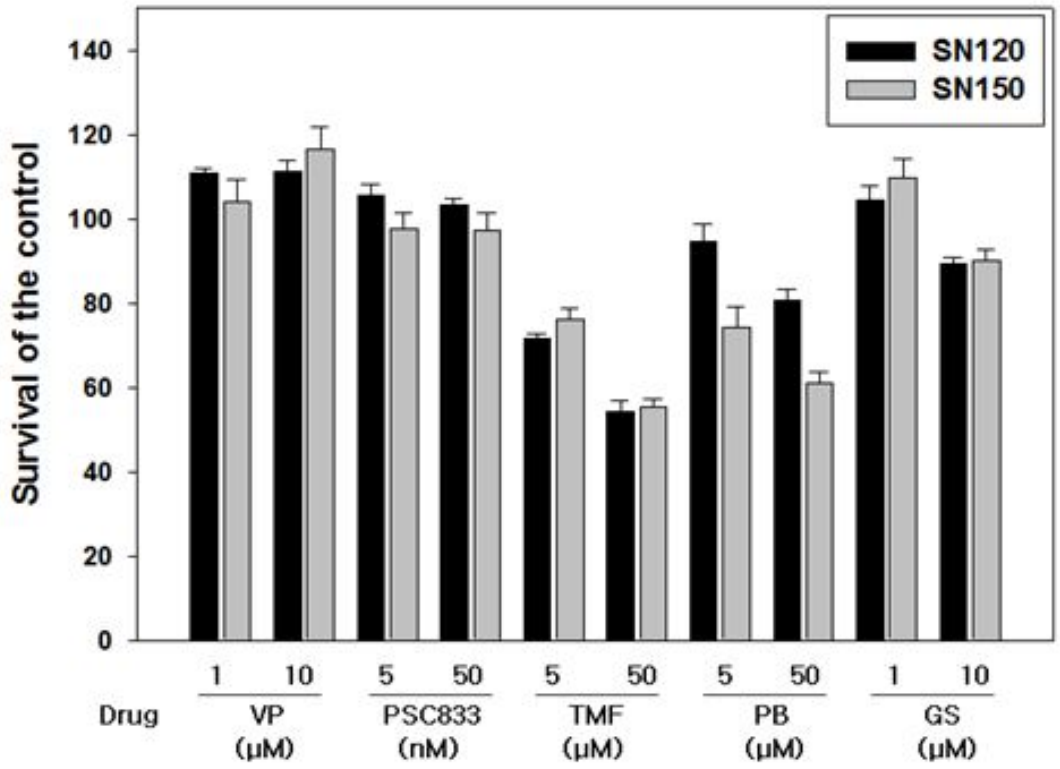
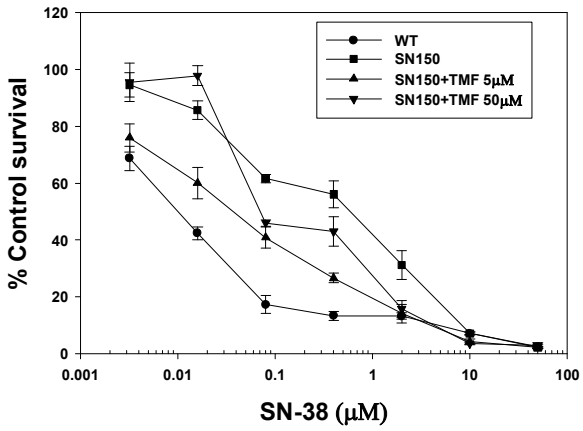



Figure 5. Sensitivity of SN38-resistant T47D sublines to SN38 in the presence of various chemosensitizers. Vp, verapamil; TMF, 3,4,5-trimethoxyflavone; PB, probenecid; GS, genistein.


WT
SN150
SN150+TMF 5μM
SN150+TMF 50μM
% Control survival
SN-38 (μM)
120
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100

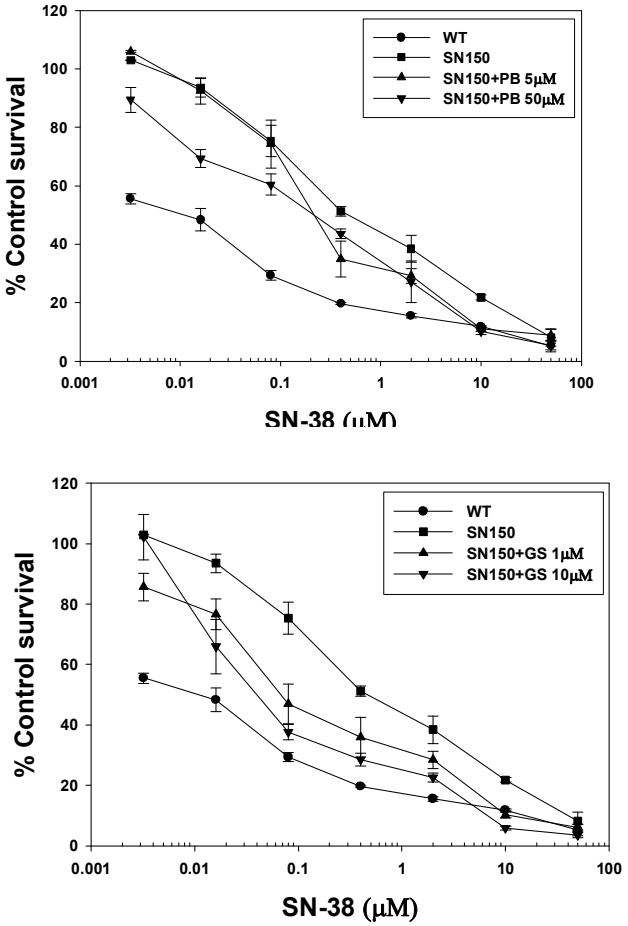



Figure 6. Sensitivity of the T47D/WT, T47D/SN120 and T47D/SN150 cells to SN38 in the presence of various chemosensitizers. Vp, verapamil; TMF, 3,4,5-trimethoxyflavone; PB, probenecid; GS, genistein. Sensitivity was determined by the MTT assay.

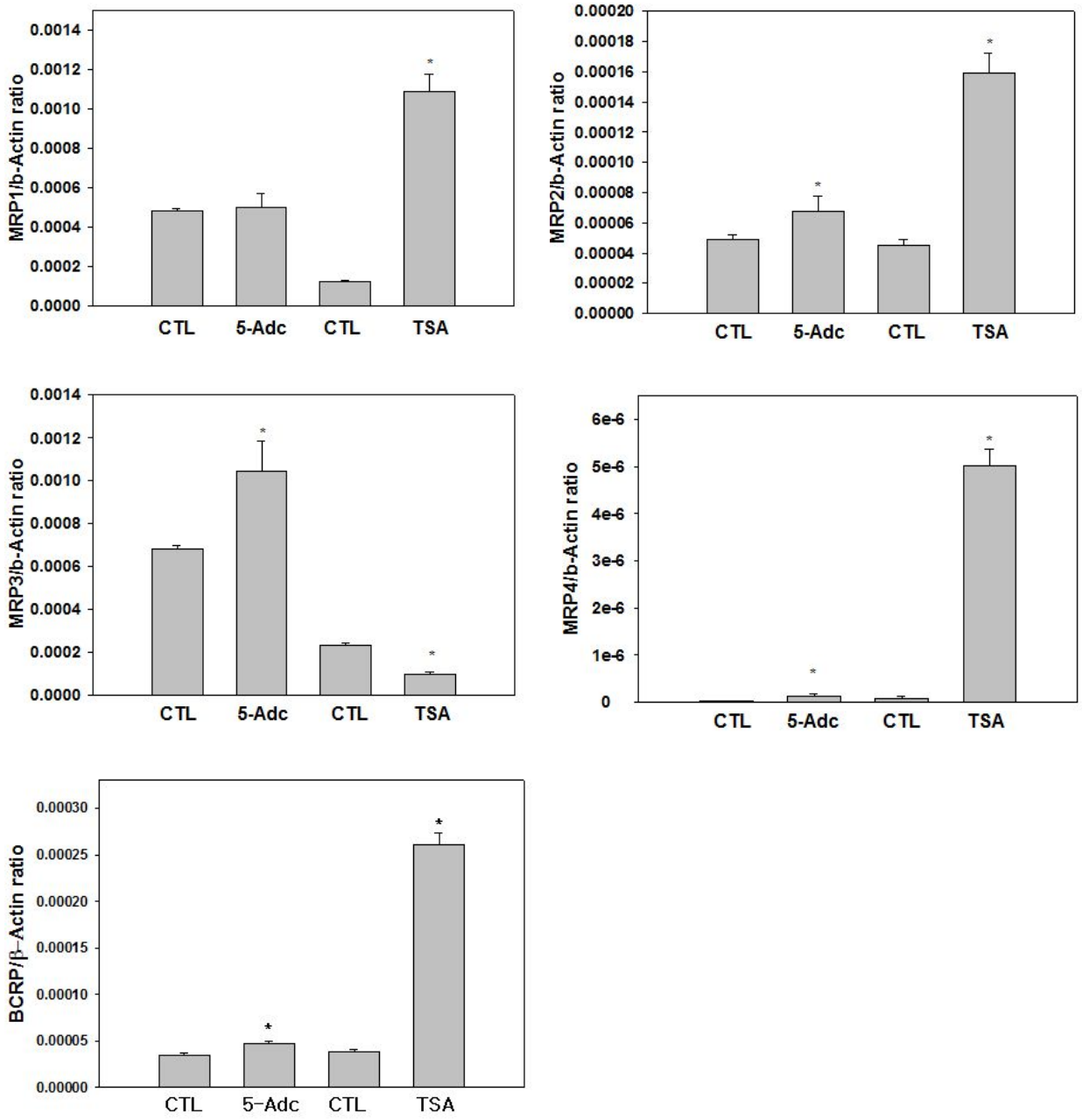



Figure 7. Effects of 5-aza-2'-deoxcytidine (AdC) and trichostatin A (TSA) on expression of MRP1, MRP2, MRP3, MRP4 and BCRP in T47D/WT cells.

*, P < 0.05 vs CTL (n=4).

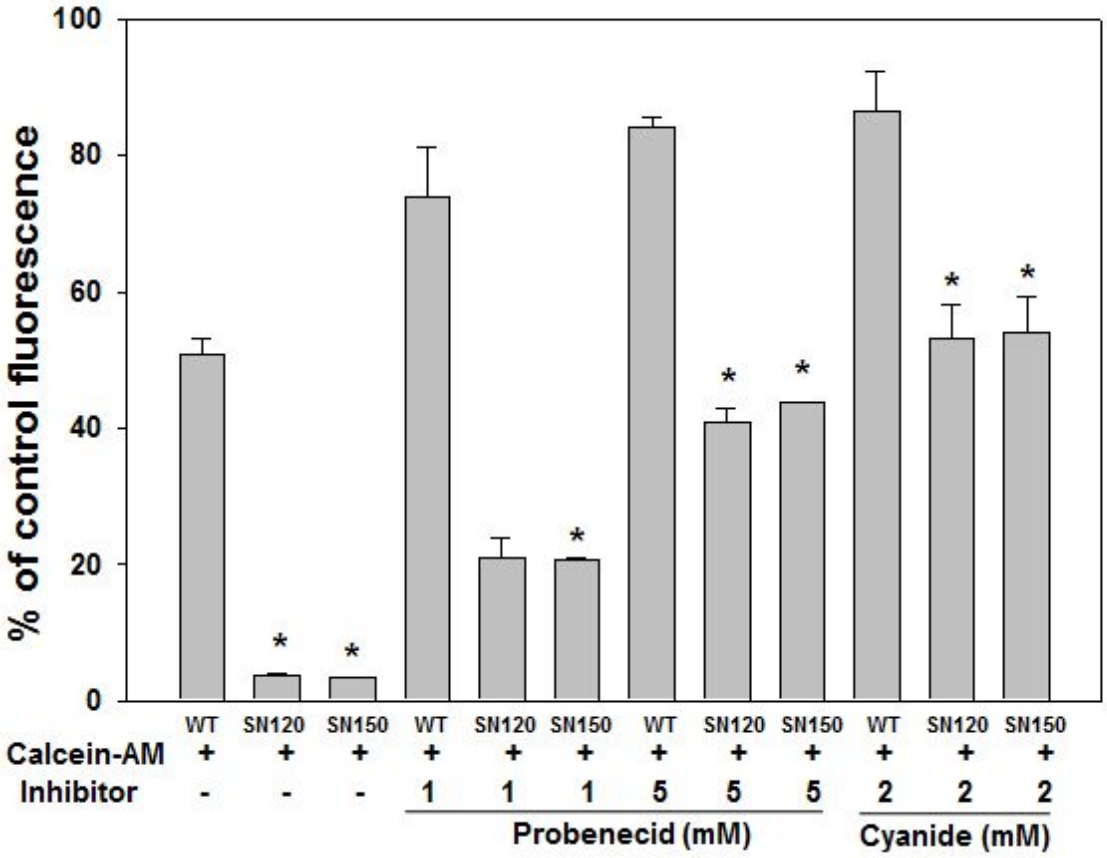



Figure 8. Intracelluar calcein-AM accumulation of the T47D/WT, T47D/SN120 and T47D/SN150 cells in the presence and absence of MRP inhibitor probenecid and ATP deplete cyanide. *, P < 0.05 vs WT (n = 3).

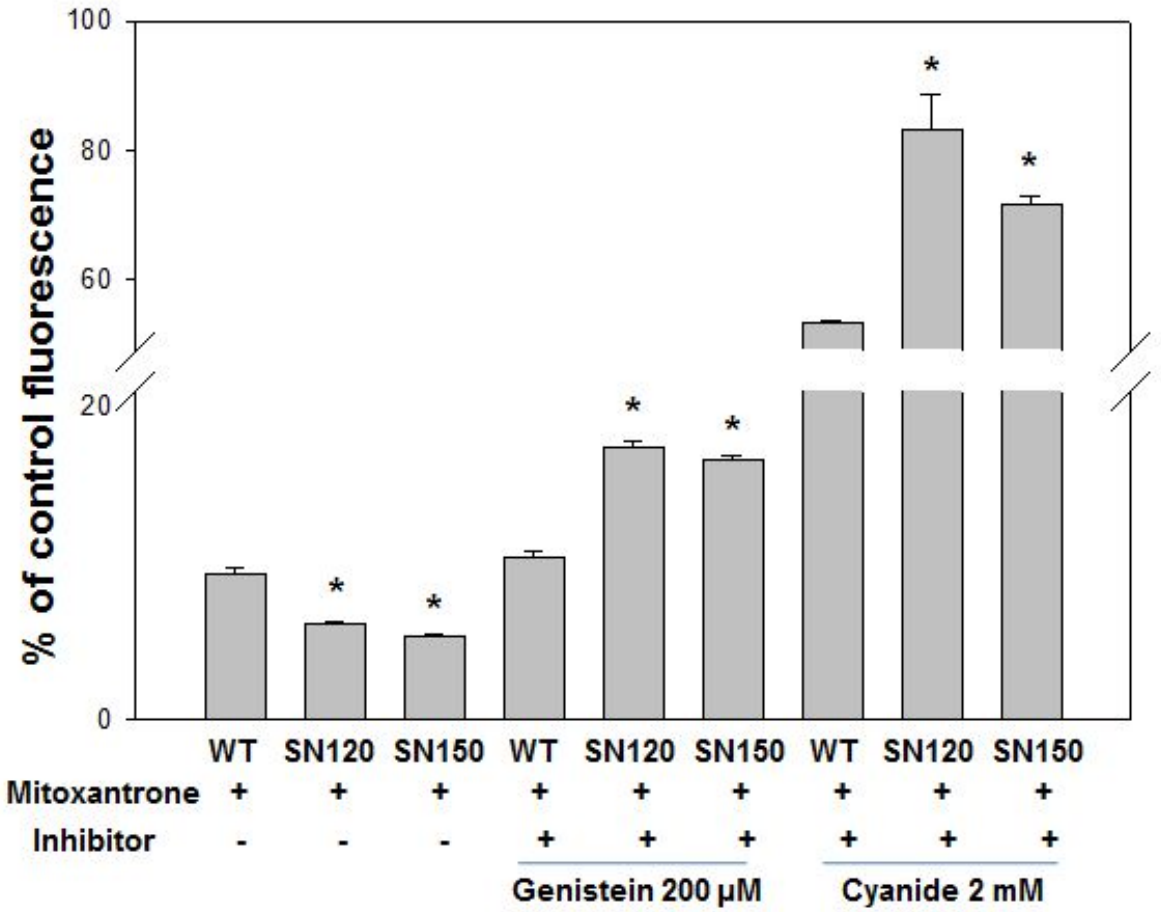



Figure 9. Intracelluar mitoxantrone accumulation of the T47D/WT, T47D/SN120 and T47D/SN150 cells in the presence and absence of chemosensitizers such as BCRP inhibitor genistein and ATP deplete cyanide. *, $P < 0.05$ vs WT ($n = 3$).

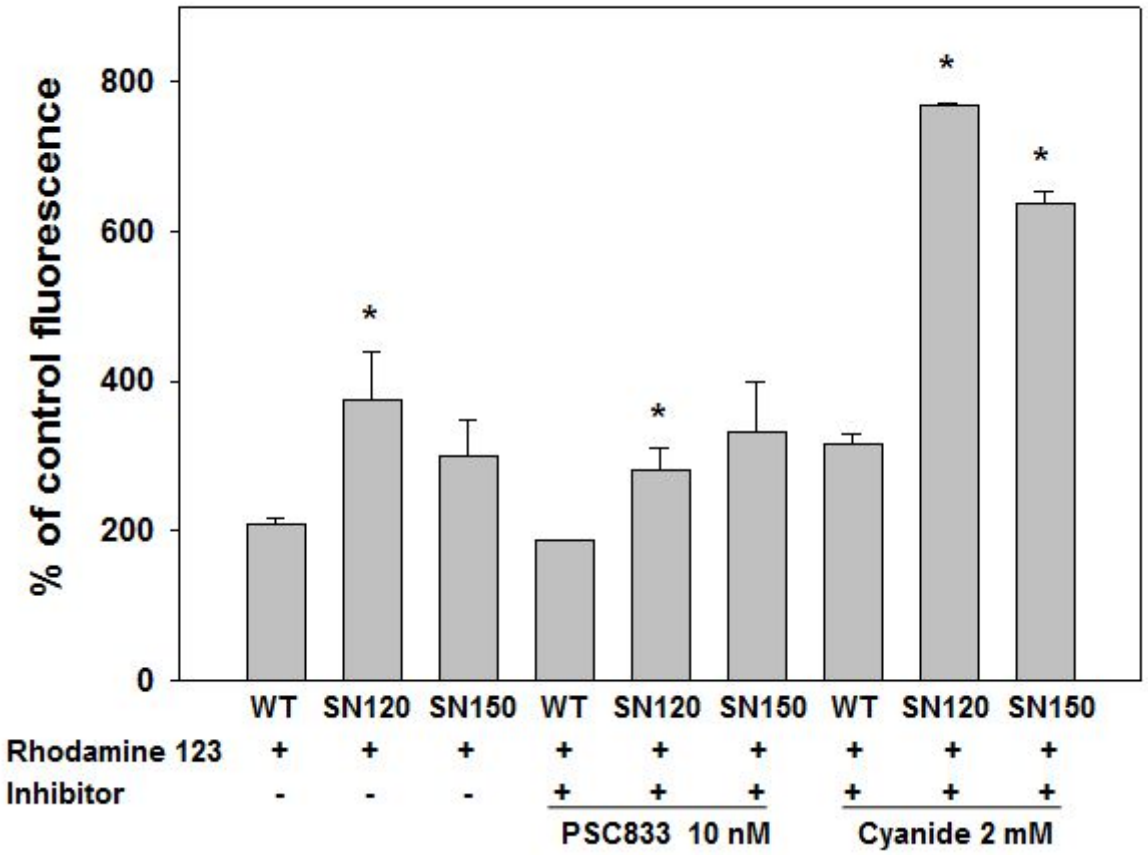



Figure 10. Intracelluar rhodamine-123 accumulation of the T47D/WT, T47D/SN120 and T47D/SN150 cells in the presence and absence of chemosensitizers such as Pgp inhibitors PSC833 and ATP deplete cyanide. *, $P < 0.05$ vs WT ($n = 3$).

# 저작물 이용 허락서

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| 연락처 | E-MAIL : hjangel21c@hanmail.net | | | | |
| 논문제목 | 한글 : BCRP와 MRP1, MRP2, MRP3, MRP4를 과발현하는 SN38 내성 T47D 유방암 아세포주의 특성<br>영어 : Characterization of SN38-resistant T47D breast cancer cell sublines overexpressing BCRP, MRP1, MRP2, MRP3, MRP4 | | | | |

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제,
   기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만,
   저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한
   권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의
   전송·출력을 허락함.

**동의여부 : 동의( O ) 반대( )**

2014 년 8 월 25 일

저작자: 이희정 (서명 또는 인)

## 조선대학교 총장 귀하