Novel strategies for targeted therapy in NETs: Inhibition of Wnt signaling in neuroendocrine tumors and Improving peptide receptor chemoradionuclide therapy (PRCRT) by a combination of 5-fluorouracil and epigenetic modifiers

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Eidesstattliche Erklärung

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List of Publications during MD:

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(3) **Xi-Feng Jin,** Spöttl G, Maurer J，Christoph J. Auernhammer.**Inhibition of Wnt/beta-catenin signaling by the porcupine inhibitor Wnt974 in neuroendocrine tumors in vitro – antitumoral effects**. *submitted* to peer reviewed international journal

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Abstracts:

(1) **Jin XF**[A], Spoettl G[A] Maurer J[A] Auernhammer C J[B]

The Porcupine (PORCN) Inhibitor WNT974 Exerts Multiple Anticancer Activities in Neuroendocrine Tumor Cell Lines in Vitro.

16th Annual ENETS Conference for the Diagnosis and Treatment of Neuroendocrine Tumor Disease, 6-8 March 2019, Barcelona, Spain.

(2) **Jin XF**[A], Auernhammer C J[A], Ilhan H[B], Lindner S[B], Nölting S[A], Maurer J[A], Spoettl G[A], Orth M[C].

"Combination of 5-fluorouracil (5-FU) with DNMTs or HDACs as Epigenetic Modifiers has Synergistic Effects on Radiosensitization and Somatostatin Receptor sstr 2 Expression in Human Neuroendocrine Tumor Cells."

16th Annual ENETS Conference for the Diagnosis and Treatment of Neuroendocrine Tumor Disease, 6 - 8 March 2019, Barcelona, Spain

(3) **Xi-Feng Jin**[1], Christoph J. Auernhammer[1,2], Harun Ilhan [2,3], Simon Lindner[3], Svenja Nölting[1,2], Julian Maurer[1], Gerald Spöttl[1], und Michael Orth[4,5,6]

Die Kombination von 5-Fluorouracil und epigenetisch wirksamen Wirkstoffen erhöht Radiosensitivität, Somatostatinrezeptor-2 Gen-expression und Radi-oligandenbindung in neuroendokrinen Tumoren *in vitro*

Jahrestagung der Deutschen Gesellschaft für Radiooonkologie (Annual Meeting of German Society for Radiation Oncology (DEGRO), 13-16. June 2019, Münster, Germany

Zusammenfassung

Neuroendokrine Tumoren (NET) sind seltene Tumoren mit häufiger Primaruslokalisation im GastroEnteroPankreatischen System und in der Lunge. Die Behandlungsstrategie der NET hängt von der Tumorlokalisation, dem Grading, dem Staging und der hormonellen Funktionalität ab. Durch eine Operation können NET bei frühen lokalisierten Erkrankungsstadien kurativ behandelt werden.

Die palliative Therapie von fortgeschrittenen inoperablen NETs umfasst verschiedenen Systemtherapieansätze wie Biotherapie mit Somatostatin-Analoga und Interferon-alpha, Peptid Rezeptor Radionuklid Therapie (PRRT), Chemotherapie oder molekular zielgerichtete Therapieoptionen . In den letzten Jahren wurden signifikante Verbesserungen bezüglich der Tumorkontrolle in der Systemtherapie von NET erreicht, jedoch besteht weiterhin ein Bedarf die molekularbiologischen Mechanismen der Tumorzelle vertieft zu verstehen und neuartige Behandlungsstrategien für eine weiter verbesserte Systemtherapie von NET zu entwickeln. Vor diesem Hintergrund wurden im Rahmen der hier vorgelegten Dissertationsarbeit zwei neue Strategien der “targeted therapy” in humanen NET Zelllinien in vitro untersucht:

Kapitel 1:

Hintergrund und Zielsetzung: Der Wnt/β-Catenin Signalweg spielt eine wichtige Rolle bei verschiedenen Malignomen. Strategien zur “targeted therapy” des Wnt/β-Catenin Signalwegs sind aktuell in klinischer Entwicklung. Bei NET ist die Rolle des Wnt/β-Catenin Signalweg bisher nur unvollständig untersucht-so reguliert beispielsweise das Tumorsuppressorprotein Menin (Gen MEN1) in NET Zellen den Abbau von Beta-Catenin. Die hier vorgelegte Studie untersuchte die Effekte des Porcupine (PORCN) Inhibitors WNT974, des direkten β-Catenin-Inhibitors PRI724 und von β-Catenin small interfering (si)RNAs in den humanen neuroendokrinen Tumor (NET) -Zelllinien BON1, QGP und H727 in vitro.

Methoden: Die NET-Zellen wurden mit WNT974 oder PRI724 inkubiert oder mit β-Catenin siRNAs transient transfiziert und anschliessend jeweils Zellproliferationsassays, Durchflusszytometrie, Caspase3/7-Test und verschiedene Western-Blot-Analysen durchgeführt.

Ergebnisse: Die Behandlung von NET-Zellen mit dem PORCN-Inhibitor WNT974 inhibierte die Zellvitalität auf dosis- und zeitabhängige Weise signifikant. Analog führte auch die Inkubation verschiedener NET-Zelllinien mit dem β-Catenin-Inhibitor PRI724 zu einer signifikanten Wachstumshemmung. Der siRNA-vermittelte Knockdown der β-Catenin-Expression reduzierte die Vitalität der BON1-Tumorzellen, jedoch nicht der H727-Tumorzellen. In den untersuchten NET Zelllinien inhibierte der PORCN Inhibitor WNT974 in Westernblot-Analysen nicht

nur das “canonical Wnt signaling” (zum Beispiel: LRP6 Rezeptor, β-Catenin und downstream targets wie c-Myc und cyclinD1) sondern auch das “non-canonical Wnt signaling” (zum Beispiel pAKT/mTOR-, pEGFR- und pIGFR-Signaling).

Zusammenfassung und Schlussfolgerung:

Der Porcupine (PORCN) Inhibitor WNT974 oder der direkte β-Catenin-Inhibitor PRI724 führten in NET Zellinien in vitro zu einer spezifischen Hemmung des Wnt/β-Catenin Signalwegs und signifikanten antiproliferativen Effekten. Weitere präklinische und klinische Studien zur Untersuchung der möglichen Rolle einer Inhibition des Wnt/β-Catenin Signalwegs als “targeted therapy” bei NET sollten angestrebt werden.

Kapitel 2:

Hintergrund und Zielsetzung: Zur weiteren Effektivitätssteigerung der Peptid Rezeptor Radionuklid Therapie (PRRT) in der Therapie von NET wird aktuell die sogenannte Peptid Rezeptor Chemo Radio Therapie (PRCRT) in präklinischen und klinischen Studien evaluiert. Die hier vorgelegte Studie untersuchte die Effekte von 5-Fluorouracil (5-FU) jeweils in Kombination mit dem DNA Methyltransferase Inhibitor Decitabin (DEC) und dem Histon Deacetylase Inhibitor Tacedinalin (TAC) in humanen neuroendokrinen Tumor (NET) -Zelllinien BON1, QGP und H727 in vitro.

Methoden: Die humanen NET-Zelllinien BON1 und QGP1 wurden mit 5-FU alleine

oder in Kombination mit DEC oder TAC behandelt. Die Radiosensitivität der Tumorzellen wurde nach Inkubation mit den jeweiligen Testsubstanzen mit Gamma-Bestrahlung (γ-IR) in Dosierungen von 0, 2, 4 oder 6 Gy getestet. Die Somatostatinrezeptor Typ 2 (SSTR2) -Expression und die [68Ga] Ga-DOTA-TOC-Radioligandenbindung der NET-Zelllinien wurde untersucht. Zellproliferationsassay, Colony-Formation-Assay, Western-Blot-Analyse und Radioligandenbindungsassay wurden hierzu angewandt.

Ergebnisse: Die Behandlung mit 5-FU plus DEC oder TAC hemmte die Vitalität der Tumorzellen im Zellproliferationsassay und regulierte die Expression von Zellzyklusmarkern sowie von pro- und antiapoptotischen Proteinen – so wurden zum Beispiel aktivierte Caspase-3, cleaved-PARP und p21 induziert, während Bcl-2, CHK1, CyclinD1, CDK1 inhibiert wurden. 5-FU allein oder in Kombination mit DEC oder TAC steigerte jeweils die Radiosensitivität der Tumorzellen signifikant, wobei in BON1 Zellen Sensibilisierungsverstärkungsverhältnisse (SER) von 2,19, 3,96 und 9,46 und in QGP1 Zellen Sensibilisierungsverstärkungsverhältnisse (SER) von 1,20, 1,31 bzw. 1,87 bestimmt wurden. Die Behandlung mit 5-FU allein oder plus DEC oder TAC induzierte signifikant die Somatostatinrezeptor-Typ-2-Expression (SSTR2) der Tumorzellen und verursachte in vitro eine stärkere Bindung des Radioliganden 68Ga-DOTATOC an die Tumorzellen.

Zusammenfassung und Schlussfolgerung: Die Kombination von 5-FU mit epigenetischen Modifiern wie DNMT Inhibitoren oder HDAC Inhibitoren zeigte am Model von humanen NET Tumorzellen in vitro: i) antiproliferative Effekte, ii) Radiosensibilisierung iii) Steigerung der Expression der Somatostatin Rezeptor Typ 2 (SSTR2) Expression, iv) Steigerung der Radioligandenbindung von 68Ga-DOTATOC. Diese präklinischen in vitro Ergebnisse legen nahe, dass eine PRCRT mit 5-FU in Kombination mit epigenetischen Modifiern wie DNMT Inhibitoren oder HDAC Inhibitoren eine vielversprechende mögliche Strategie zur weiteren Verbesserung der Wirksamkeit der PRRT bei der Behandlung von NETs sein könnte. Weitere präklinische und klinische Studien sollten angestrebt werden.

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Introduction

Novel strategies for targeted therapy in NETs:

Inhibition of Wnt signaling in neuroendocrine tumors and Improving peptide receptor chemoradionuclide therapy (PRCRT) by a combination of 5-fluorouracil and epigenetic modifiers

Neuroendocrine tumors (NET), neoplasms frequently occur in the intestine, pancreas, lung, or other parts of the human body[1-2]. NET treatment depends on primary tumor localization, histopathological classification and grading, staging, and hormone secretion [3-6].

Treatment of NETs, like in most other human cancers, includes surgical resection, if diagnosed at the early stage of disease. Surgery can cure NET in early localized disease or debulking surgery can relieve symptoms and improve survival in distant metastasized disease[7-8]. Systemic treatment options in advanced inoperable disease include chemotherapy, targeted therapy with the mTOR inhibitor everoli-

mus and the multi-tyrosine kinase inhibitor sunitinib, biotherapy with somatostatin analogues and interferon alpha, and peptide receptor radionuclide therapy (PRRT) [9-11]. Over the last decade, significant improvements have been made for the treatment options and clinical outcomes of NET. However, further investigations are needed to better understand the molecular mechanisms and to develop novel treatment strategies to control NET growth.

In this doctoral thesis, the first chapter of the research is focusing on Wnt signaling pathways, which are involved in the wnt canonical/beta catenin pathway and non-canonical pathway. The selective porcupine (PORCN) inhibitor WNT974 has exerted therapeutic potential in various preclinical cancer models and clinical trials, and also displayed radiosensitizing or chemosensitizing effects [11-17].We further explored WNT974 anticancer properties in NET cells. We expected to provide evidence regarding the wnt signaling pathway in NET pathogenesis and to develop WNT974 as a potential strategy to control NET.

In this doctoral thesis, the second chapter of the research is to investigate the

synergistic effects of 5-fluorouracil (5-FU) combined with DNA metlhyltransferase (DNMT) inhibitors or histone deacetylase (HDAC) inhibitors as epigenetic modifiers on radiosensitization and somatostatin receptor 2 (SSTR2) expression and binding in human NET cells.

The PRRT uses a radiopharmaceutical that targets the peptide receptors to deliver localized radiotherapy to NET cells and to date, [177 Lu-DOTA0, Tyr3]-octreotate (177 Lu-octreotate) is the most frequently used PRRT radiopharmaceutical with highly efficient results regarding disease control rate(DCR), PFS as well as OS [18-21]. However the ORR of NETs following PRRT is limited to 20-30% [18-21]. The essential prerequisite for PRRT of NETs is the high abundance of the peptide receptor somatostatin receptor type 2 (SSTR2) on the NET cell surface vs. normal cells [22-23].

The complete remission of patients with metastasized NET after the 177 Lu-octreotate PRRT is still difficult; thus, additional therapy or targets are needed to improve PRRT efficacy on NETs. Indeed, previous studies showed that the

combined chemotherapy with PRRT could offer better potential in effective control of NETs [24-25], and PRCRT (peptide receptor chemoradionuclide therapy) using the concomitant infusion of 5-fluorouracil (5-FU) in comparison to PRRT improved treatment responses and OS of patients with NETs [26]. Moreover, combination of capecitabine and temozolomide with 177 Lu-octreotate resulted in better PFS of patients with metastasized NETs and only modest adverse effects [27].

Furthermore, DNA methylation/demethylation, and histone acetylation/deacetylation are fundamental factors in genetic modifacations [28-30]. HDAC inhibitors have been developed to treat various human cancers [31-32]. Indeed, previous studies revealed that the DNMT inhibitor decitabine(DEC) in combination with an HDAC inhibitor tacedinaline(TAC) could synergistically upregulate expression of somatostatin receptor type 2 (SSTR2), a target of the PRRT, in NETs [33]. Other preclinical studies reported that combinations of 5-FU with HDACi or DNMTi causes synergistic antitumoral effects in gastric, pancreatic, and colon cancer cells [34]. Thus we combined 5-FU with DEC or TAC tand investigated whether these

combinations might cause synergistic effects on radiosensitzation, SSTR2 expression and specific radiologand binding on NET cells. We expected to gain important preclinical information on novel strategies how to increase the efficacy of PRCRT on NETs.

In this doctoral thesis,

- data presented in chapter 1 will reveal that the PORCN inhibitor WNT974 concentration and time-dependently inhibited NET cell proliferation, induced G1 or G2 arrest but not apoptosis.WNT974 blocked not only beta catenin dependent but also non-canonical Wnt/receptor tyrosine kinase pathways (e.g, PI3K/AKT/mTOR, pEGFR/EGFR and pIGFR/IGFR signaling) in NETs (beta-catenin inhibitor PRI724 as well).
- data presented in chapter 2 will demonstrate 5-fluorouracil (5-FU) in combination with the DNA methyltransferase(DNMT) inhibitor decitabine (DEC) and the histone deacetylase (HDAC) inhibitor tacedinaline (TAC), to i) suppress cell viability and induce apoptosis, ii) to cause radiosensitization in

NET cells, at least in part, due to a massively decelerated efficacy of DNA damage repair; iii) to upregulate the expression of SSTR2 and iv) to increase radioligand binding of 68Ga-DOTA-TOC in NET cells. Thus this study suggest that combinations of 5-FU with epigenetic modifiers might constitute suitable approaches to enhance the efficacy of PRRT by combining the radiosensitizing potentials of these drugs on one hand and the upregulation of SSTR2 expression and thus, of radioligand binding on the other.

Taken together, the presented date demonstrate

i) blocking Wnt signaling as a novel strategy for targeted therapy in NETs,

ii) combination of 5-fluorouracil and epigenetic modifiers to enhance antiproliferative effects, radiosensitization,SSTR2 expression and specific radioligand binding in NETs and thus being of potential strategy for peptide receptor chemoradiation therapy (PRCRT).

Future preclinical and clinial trials are required to further investigate the therapeutic efficacy of these novel strategies for NETs.

1 Chapter one: Inhibition of Wnt signaling by porcupine inhibitor WNT974 in neuroendocrine tumors *in vitro*-antitumoral effect

1.1 Introduction

The Wnt/beta-catenin signaling cascade play important roles in various cancers [35-37]. Wnt/beta-catenin signaling has been revealed to drive tumor cell proliferation [38-41] and promote cell invasion and migration [42].

Thus, our research is focusing on the Wnt signaling pathways, which are a group of signal transduction proteins that transmit cell surface receptor signaling into the cell nucleus, resulting in gene expression and cell proliferation and migration during embryo development and tumorigenesis, including NET [43-46]. The Wnt signaling can be further classified into canonical wnt, noncanonical planar cell polarity,noncanonical/receptor tyrosine kinases and noncanonical/calcium pathways, all mediated by wnt ligand binding to a Frizzled family receptor [35].

In the wnt-off state (Figure.1-1), β-catenin is phosphorylated by serine/threonine

kinase casein Kinase (CK) or GSK-3, facilitated by scaffolding proteins APC and Axin (forming the degradation complex or destruction complex) and could be recognized by β-TrCP, then finally ubiquitinated for degradation via proteasome. Beta-catenin dependent cascade [47-49] is triggered when the secreted Wnt proteins (such as Wnt1 or Wnt3) bind to the FZDs (Frizzled receptors 1-10) as well as the LRP5/6 which lead to phosphorylatation of dishevelled (DVL) and further inducing the interaction of DVL with Axin, Thereafter, unphosphorylated beta-catenin will accumulate and transfer into the nucleus, and bind to TCF/LEF family to activate its targeted gene transcription and expression (eg. cyclinD1/D3, c-Myc) [50-59].

In contrast, non-canonical Wnt signaling is primarily triggered by Wnt5a, wnt5b and Wnt11 [43-46], which were further mediated by ROR1/ROR2 and RYK, exerting reciprocal effect with the canonical way (Fig.1-2) [47-49,52-59].

Many small molecules [60-66] targeting the wnt siganaling pathway such as Dvl inhibitors, beta-catenin destruction complex inhibitors (GSK3 inhibitor, CK1 inhibitor), direct beta-catenin inhibitors, PORCN (an o-acetyltransferase, palmitoylation

of wnt protein [67-68] inhibitors, tankyrase(TNKS) inhibitors and TCF/LEF inhibitors have demontsrated promising anti-tumor effect, especially porcupine inhibitor WNT974 as well as beta-cetenin inhibitor PRI724 have already entered clinical trials. WNT974 has shown therapeutic potentials in various preclinical models, by suppressing the cell viability and distant metastasis or invasion[43-46]. also by serving as a chemosensitizer [69] ,immunotherapy modulator or radiosensitizer [70].

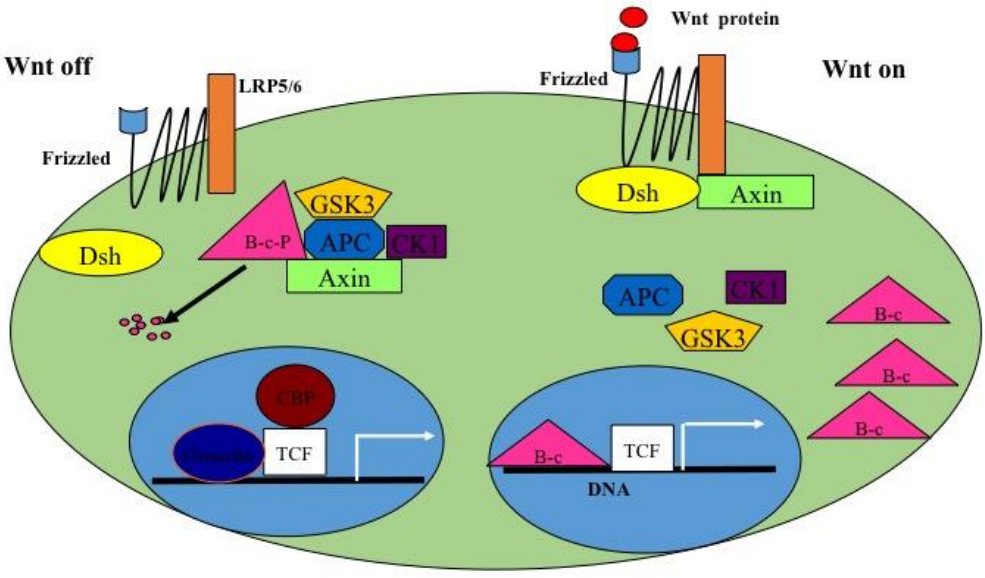

Fig. 1-1. Wnt carnonical /β-catenin pathway (adapted from reference 52-59)**.**

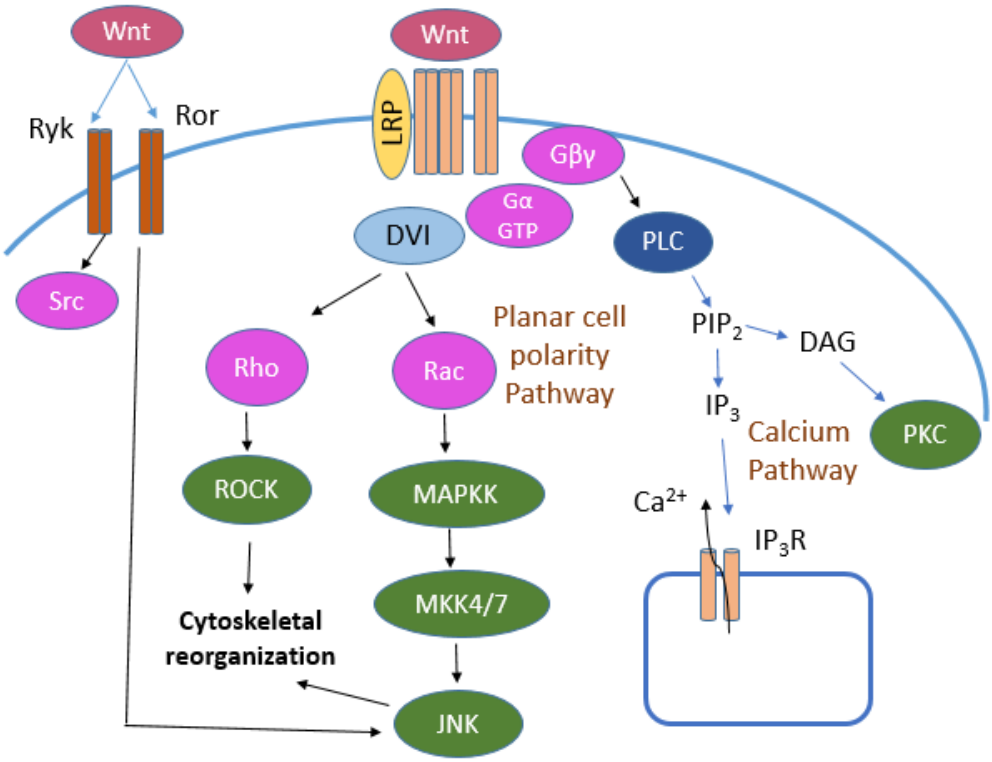

Fig.1-2. Non-canonical Wnt signaling pathway (adapted from reference 52-59).

Moreover, the wnt/β-catenin antagnist PRI724 specically inhibit the binding of β-catenin with the coactivatior CREB-binding protein(CBP), and PRI-724 also enters the clinical studies for colon cancer or liver fibrosis [71-72].

menin has a high frequency of mutations in pNETs [38,73].When the β-catenin

was knockout in Men1 deficient PNETs, the tumor was inhibited and the survival time was prolongated in vivo. Some retrospective analysis of human SI-NETs [74,75] and pNETs corfirmed the upregulation of wnt signaling as well as the loss of APC.

To sum up, much still needed to be explored in the NETs regarding wnt sigalling pathway. In this study, we gained further insight into the wnt canonical and non-canonical signaling cascade in NET cells and highlighted the development of therapeutic targeting the wnt signaling moleculars(PORCN inhibitors and be-ta-catenin inhibitors) in NETs treatment.

1.2 Materials and methods

1.2.1 cell types and reagents

All the cell lines(see Table 1-1) were verified the identities via short-tandem repeat (STR) sequences by DSMZ (German Biological Resource Centre) [76].WNT974 (synonym:LGK974) was provided by Novartis corporate(Basel, Switzerland), PRI-724 was purchased from Selleckchem (Germany).

Cell lines	Pheno-type	Sources	Medium
QGP	pancreatic islet	Japanese Collection of Research Bioresources Cell Bank	DMEM+PBS+penicillin/streptomycin +amphotericin B
Bon1	pancreatic	Prof.R.G.ke/Marburg,Germany	DMEM+PBS+penicillin/streptomycin+amphotericin B
H727	bronchopulmonary	American Type Culture Collection ,Manassas	RPMI-1640+penicillin/streptomycin+PBS+amphotericin B
GOT1	midgut carcinoid	Prof.O.Nilsson,Sahlgrenska University Hospital Sweden	RPMI-1640+penicillin/streptomycin+FBS+amphotericin B +insulin

Table 1-1. Overview of NET cell lines(human) and cell culture.

1.2.2 Cell proliferation assay

Firstly, we grown NET cells overnight and administerested them with increasing concentrations of the agents(WNT974[1 µM-16 µM] or PRI-724[1 µM -10 µM]) for 72 h or 144 h, then add 20ul of cell titer blue reagents (Promega,USA), and futher incubated for 4 h. cell absorbance rates were determined using an GLOMAX plate reader (Promega, Madison, USA).

1.2.3 Flow cytometric cell cycle distribution assay

NET cells were grown and treated with increasing concentration (1 μM-16 μM) of WNT974 for 48 h or 72 h and then harvested through trypsinization, centrifugation and two washes in PBS. After that, the cells were dissolved in propidium iodide (Sigma-Aldrich) and then analyzed and quantified with the BD Accuri C6 Analysis machine.

1.2.4 Caspase-3 and -7 apoptosis experiment

we seeded NET cells into white-walled plates and cultured overnight, then administrated them with WNT974 (1 μM and 16 μM respectively) for 72 h and then exposed to Apo-One homogeneous caspase-3/7 regeants (Promega). Firstly, mixed the Caspase-Glo®3/7 buffer with the substratethoroughly, then removed white-walled 96-well plates out of the incubator, dispensed 100 μl of well-mixed reagent per well. After incubating at RT for 2 hours, all the samples were measured by one Orion II luminometer(Pforzheim, Germany).

1.2.5 Wound healing and migration assay

we seeded NET cells into Ibidi culture-insert 2 well dishes (Ibidi, Germany) at a density of $l.2\times10^5$-1.4×10^5cells per well，then after 24 h, the insert was removed and the cell monolayer gap was created . then we treated the NET cells with various doses of WNT974. We captured and recorded the changes of the wound gap under the microscope (an Zeiss Axiovert 135 TV) equipped with a special camera (Zeiss AxioCam MRm, München, Germany) every 24 h. thereafter, NET cell migration capacity was determined by wound gap measurements at the above mentioned time point using Image J software (Bethesda, MD, USA).

1.2.6 Western blot

treated NET cells were lysed in the cell lysis buffer (Thermo Scientific, Waltham, MA, USA) and quantified. The total cellular protein samples with 50 μg per loading were segregated by SDS-PAGE, immobilized to the PVDF membranes (Millipore). For western blotting, theses blots were first blocked with clear milk blocking buffer (PIERCE, Rockford, USA) for 60 min, then incubated with a variety of primary antibodies(Table.1-2).

they were then co-incubated with HRP secondary antibodies (Cell Signaling Technology, Germany) at a dilution of 1:25000 for 4 h. The protein bands were visualized in enhanced chemiluminescence (ECL) solution followed by exposure to Amersham Hyperfilm^{TM} (WESTAR Supernova, Cyanagen, Bologna, Italy) and quantificated by ImageJ software (NIH, Bethesda, USA).

primary antibody	Company	primary antbody	Company
total β-catenin	Cell signaling #8480	CyclinD1	Cell signaling # 2978
non-p-β-catenin S45	Cellsignaling #19807	CyclinD3	Cell signaling # 2936
Ser33/37/Thr41 p-β-catenin	Cell signalin #9561	CDk1	Cell signaling # 9116
p-β-catenin S552	Cell signaling #5651	CDK4	Cell signaling#12790
Axin	Cell signaling # 2087	CDK6	Cell signaling#13331
pLRP6S1490	Cell signaling #2568	Chk1	Cell signaling #2360
LRP6	Cellsignaling #3395	EGFR	Cell signaling #4267
Dvl	Cell signaling #3224	pEGFR	Cell signaling #3777
pDvl-2	Cell signaling #3224	IGFR	Cell signaling # 3027
SFRP	Cell signaling #3534	pIGFR Y1135	Cell signaling # 3918
Wnt3a	Abcam #28472	Akt	Cell signaling #4691
Wnt5a/b	Cellsignaling #2530	pAkt S473	Cell signaling # 4060
GSK3	Cell signaling # 5676)	4EBP1	Cell signaling # 9644
pGSK3-a/ß	Cell signaling # 8566	p4EBP1 S65	Cellsignaling# 13443
JNK	Cell signaling # 9252	p70S6K	Cell signaling # 2708
pJNKT138/Y185	Cell signaling # 4668	pp70S6K T389	Cell signaling # 9205
c-Myc	Cell signaling #13987	ERK1/2	UBI # 06-182
Vimentin	Cellsignaling #5741	pERK1/2T202Y204	Cell signaling #4370
ZO-1	Cell signaling #8193	p53	Santa Cruz -126
Menin	Santa Cruz -374371	pmTOR S2448	Cell signaling #2971
cyclin B1	Cell signaling # 12231	Neurotensin	Santa Cruz -377503
S6	Cell signaling #2217	pS6 S240/4	Cell signaling #5364
Bcl-2	Abcam #694	Chk2	Cell signaling #6334
AIF	Santa Cruz -13116	E-Cadherin	Cell signaling #3195
P21 Waf1/Cip	BD #610233		

Table 1-2.Multiple primary antibodies measured in westernblot.

1.2.7 β-catenin siRNA and GSK3β siRNA transfection

We ordered small β-catenin siRNA, GSK3β siRNA or non-targeting siRNA from Dharmacon RNA Technologies(On-Target plus SMART Pool, Cat #L-003482, -003484, Cat #D-001810, respectively, Dharmacon USA). appropriate number of Bon1 and H727 cells were seeded and incubated for 16-18 h, and then subjected to optimal transfection with GSK3beta siRNA(50 nM) or β-catenin siRNA(25 nM for Bon1,and 75 nM for H727) by using DharmaFECT 2(bon1) or 3 (H727) trans-fection reagent for 72 h. then exposed to WNT974 for 72h after transfection. Thereafter, the cells were collected and subjected to cell viability and Western blotting assays.

1.2.8 Statistical analysis

Each experiments was conducted in triplicates and was repeated more than once and the data were summarized as the mean and SDs (or SEM). Statistical analysis was assessed by the student t test, Dunnett's t test and ANOVA with SPSS 16.0

software for Windows (Chicago), A p value <0.05 was considered statistically significant.

1.3 Results

1.3.1 WNT974 dose and time dependently suppressed the growth of NET cells

NET cell viability after WNT974 treatment was firstly evaluated. As shown in Fig. 1-3, WNT974 inhibited the cell viability in all three cell lines in a concentration- and time- dependent manner. At the end of 144 h incubation the cell viability was reduced to 58.20% ±12.53%, 63.99%±11.90%, 56.42%±10.84% in Bon1, QGP and H727 respectively. The IC20 values of WNT974 were 5.4 μM, 7.8 μM and 10.1 μM in Bon1, H727 and QGP respectively.

1.3.2 Suppression of NET cells migration by WNT974

We next examined the effect of WNT974 on regulation of NET cell migration capacity. The wound healing assay showed that WNT974 only suppressed the migration of Bon1 and QGP cells at the largest dosage, while showed no changes in

H727 cells (Fig.1-4). At the gene level, e-cadherin, vimentin and the tight junction protein ZO-1[77-79]were further analyzed. Though no significant change was observed in E-cadherin after WNT974 treatment, the vimentin level was markedly downregulated and ZO-1 was moderately increased in NET cells.

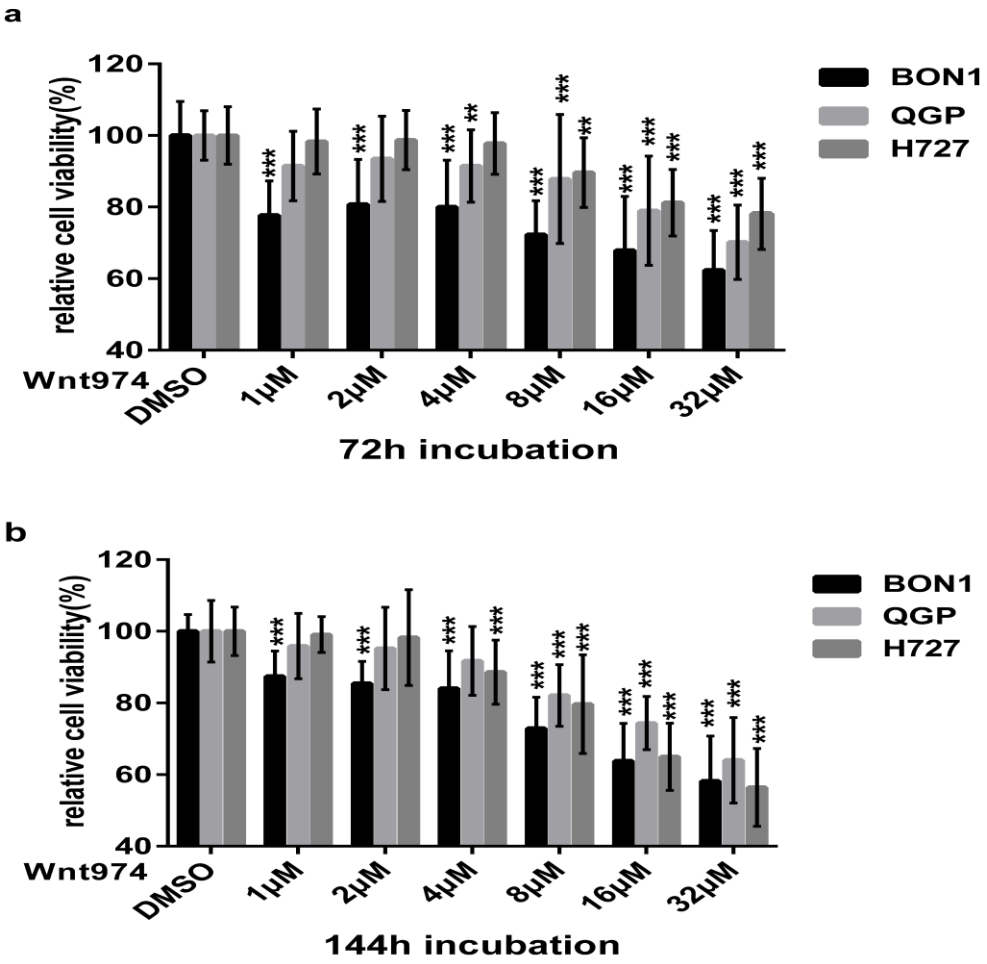

Fig. 1-3. WNT974 decreased NET cell proliferation.

(*p<0.05, **p<0.01, and ***p<0.001).

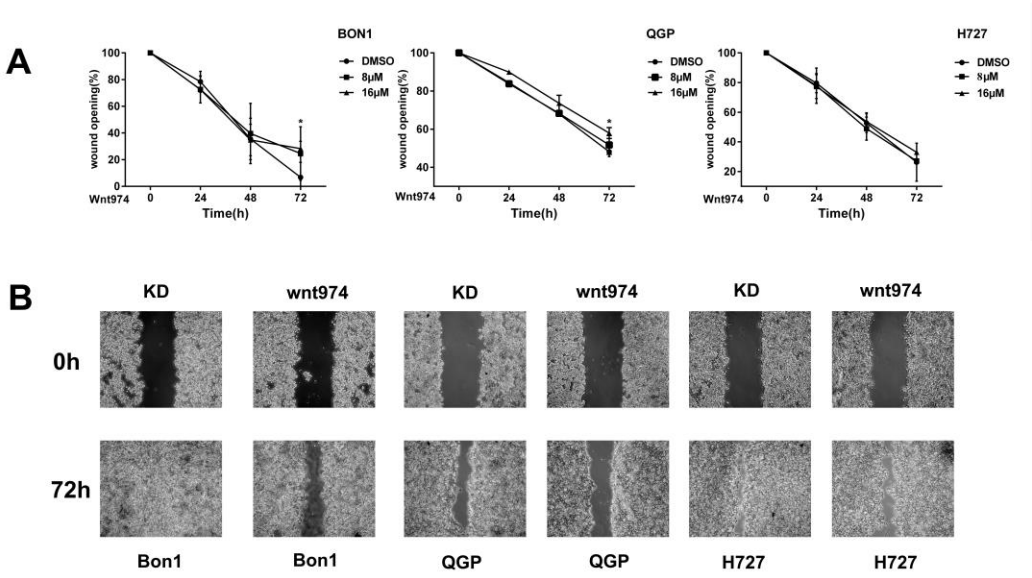

Fig.1-4. Effect of WNT974 on regulation of NET cell migration.

(wound healing assay,*p<0.05, **p<0.01, and ***p<0.001).

1.3.3 Cell cycle re-distribution after WNT974 treatment

WNT974 lead to G1 phase arrest in a dose (1 uM -16 uM) dependent mode in Bon1 and H727. The percentage of G0/G1 phase population increased from 61.4% to 75.43% in BON1, from 57.56% to 74% in H727 respectively after WNT974 treatment. Subsequently, we observed a marked reduction of S phase population, from 14.09% to 8.036%, and from 12.80% to 7.38% respectively) in these 2 cell

lines. Meanwhile the evident accumulation in the G2 phase was observed in QGP cells after WNT974 treatment (from 15.24% to 25.13%), accompanied by decreased G0-G1 phase(from 77.93% to 68.07%)(Fig. 1-5-1-8).

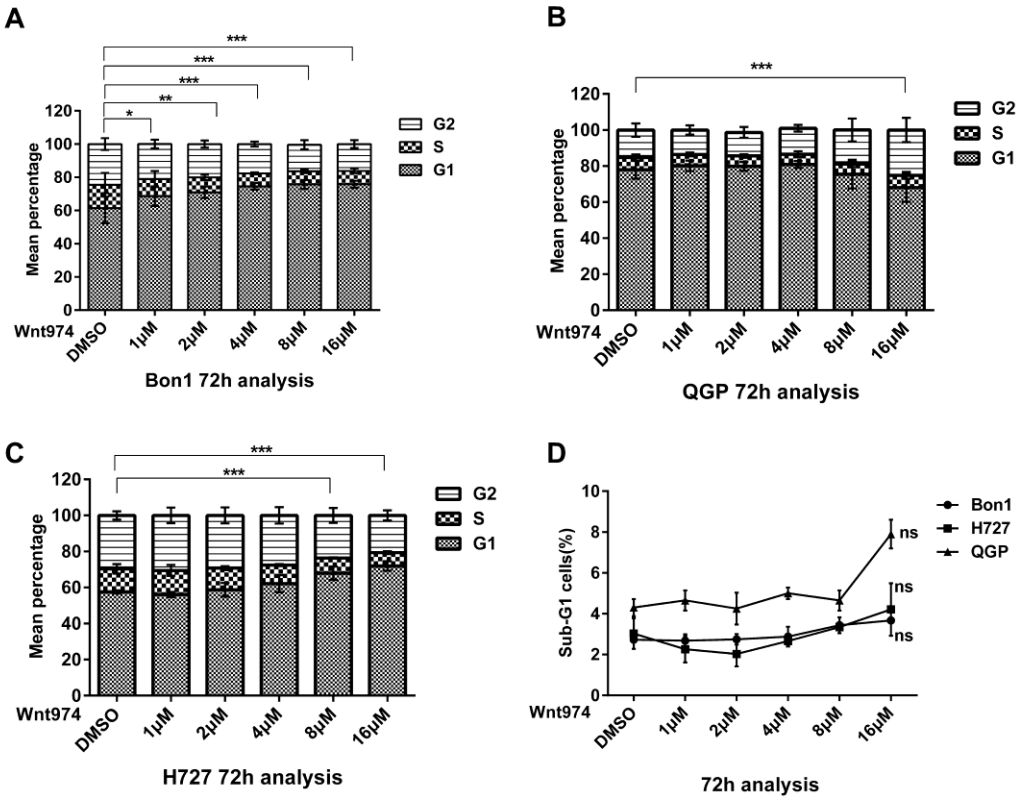

Fig. 1-5. Effect of WNT974 on cell cycle distribution.

(NET cells were treated with or without Wnt974 for 72 h,then subjected to FACS, *p<0.05, **p<0.01, and ***p<0.001).

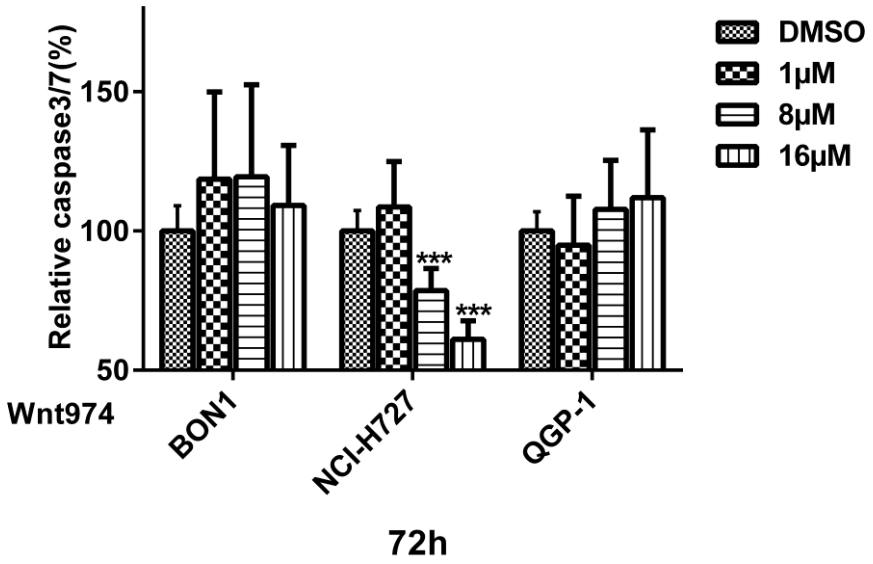

Fig.1-6. Effect of WNT974 on regulation of caspase-3/7 activity in NET cells.

(NET was treated with wnt974 for 72 h and then subjected to caspase-3/7 apoptosis assay, which shows a mean percentage of caspase 3/7 activity compared to the untreated control (100% ± SD).

Correspondingly, WNT974 reduced the expression levels of cyclins (cyclin D1/D3/B1), cyclin-dependent kinases (cdk1/4/6), and checkpoint kinase-1 (Chk1) in a dose-dependent manner. Meanwhile, WNT974 treatment reduced the expression

of cyclin kinase inhibitor proteins, like p21Cip1 and p53, although level of AIF was no significant changes in these three NET cell lines (Fig. 1-5-1-8).

On the other hand, caspase -3/7 activity assay didn't show any significant changes in apoptosis in NETs, also, the percentages of sub-G1 phase were not significantly increased (Fig.1-5-1.8). These data indicate that WNT974 antitumor activity was to induced by NET cell arrest at G1 or G2/M phase and suggest that combination of WNT974 with other apoptosis-inducers is needed to achieve the maximal an-ti-tumor activity.

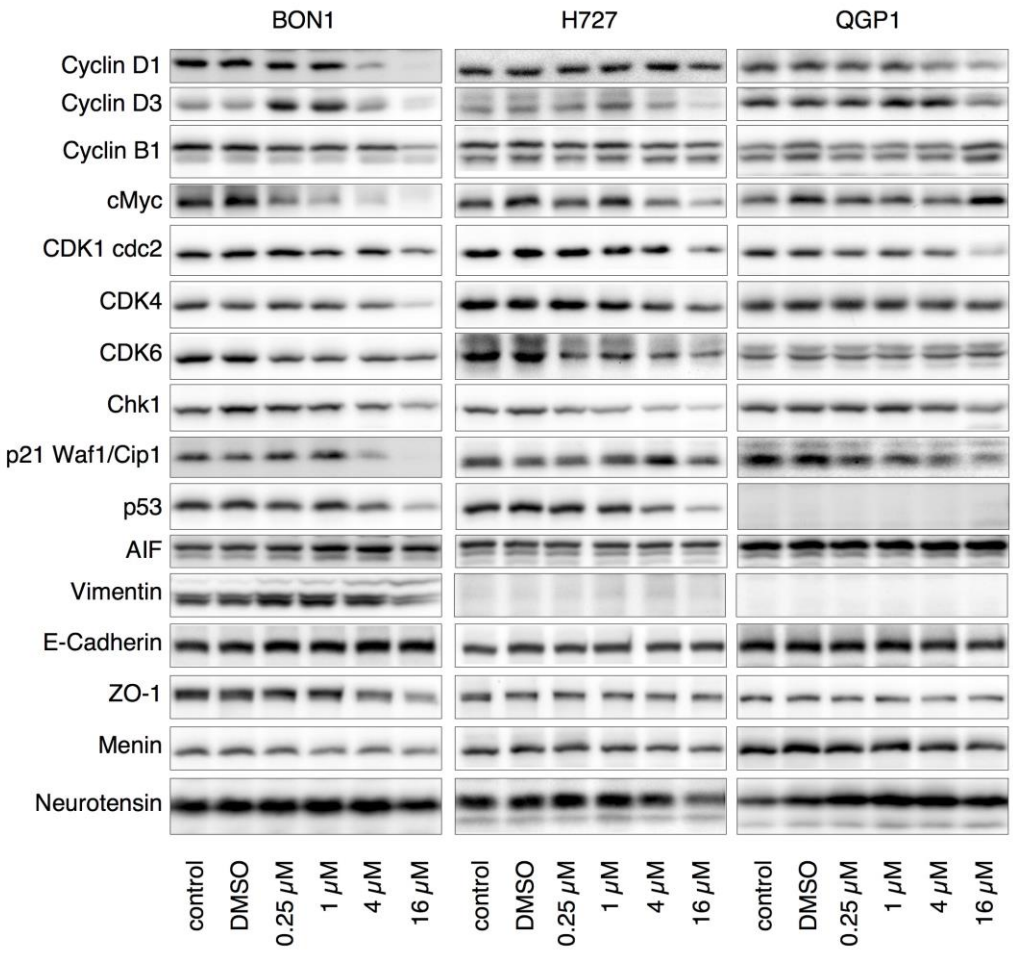

Fig. 1-7. WNT974 regulated cell cycle related proteins(western blot).

(Bon1, QGP, and H727 cells were treated with or without Wnt974 (0.25 μM to 16 μM) for 72 h and then exposed to western blotting.)

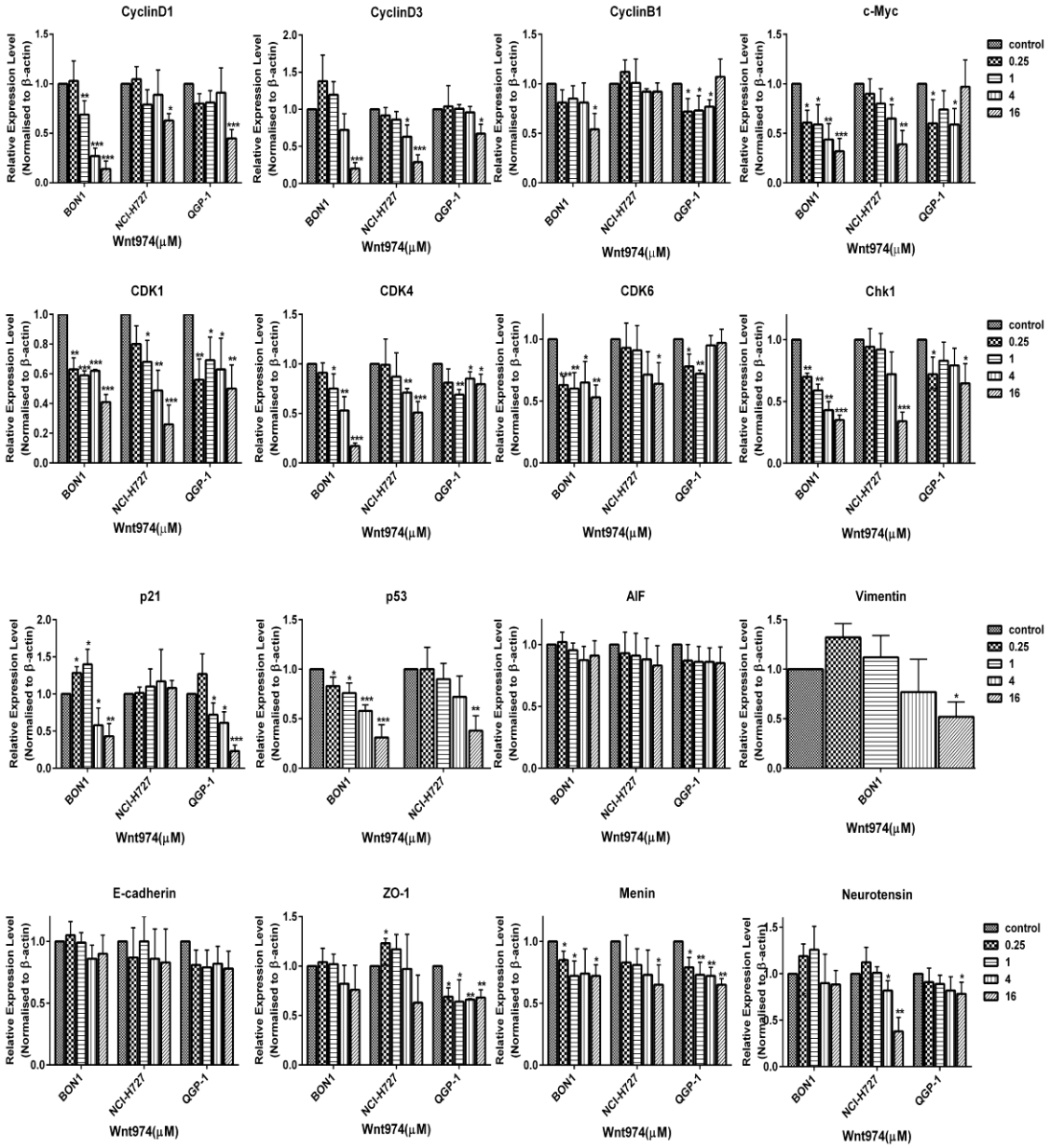

Fig. 1-8. Quantitation of western blotting of WNT974 on regulation of cell cycle markers in NET cells.(relative changes in protein bands were measured by densitiometric analysis with the control).

1.3.4 Suppression of Wnt/β-catenin signaling by WNT974

Our data showed that WNT974 treatment of NET cells downregulated expression of LRP/pLRP6 and DVL2/pDVL2, whereas WNT974 upregulated Axin in Bon1 and QGP cells and SFRP1(a antagonist of the Wnt signaling) only in H727 cells (Fig. 1-9;1-10). Levels of Wnt1, Wnt3a and Wnt5a/b were only slightly decreased (Fig.1-9;1-10). Furthermore, WNT974 treatment dose- and time-dependently reduced level of non-phosphorylated-β-catenin, total β-catenin, and related downstream targeting proteins(c-Myc/cyclinD1/cyclinD3, or others), while β-catenin phosphorylation at Ser33/Ser37/Thr41 gradually recovered back to the basal levels after 48 and 72 h treatment with WNT974 (Fig.1-9;1-10). However, these changes did not equally occur, which was obvious in Bon1, but less in H727 cells, indicating that WNT974 effects on Wnt signalling was cell type-specific.

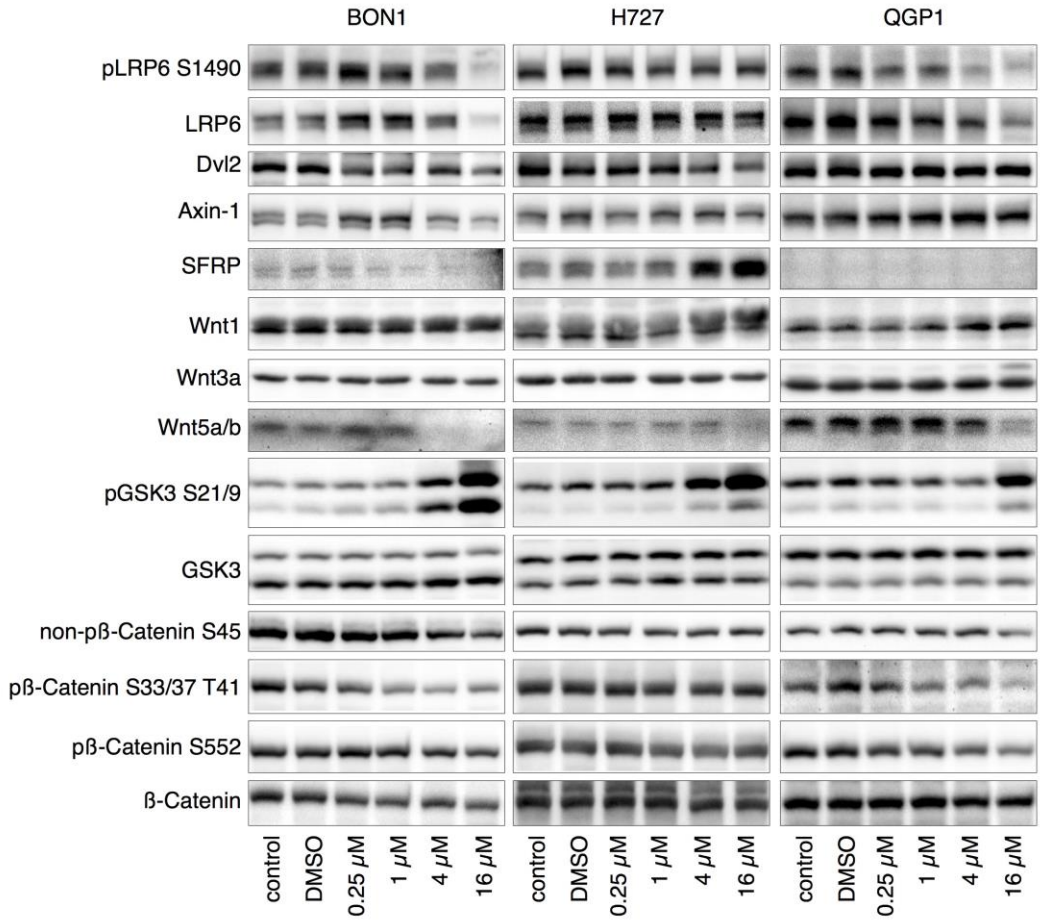

Fig. 1-9. Effects of WNT974 on inhibition of wnt/beta-catenin signaling.

Bon1, QGP, and H727 cells were treated with or without Wnt974 (0.25 µM ,1 µM, 4 µM to 16 µM) for 72 h and then exposed to western blotting.

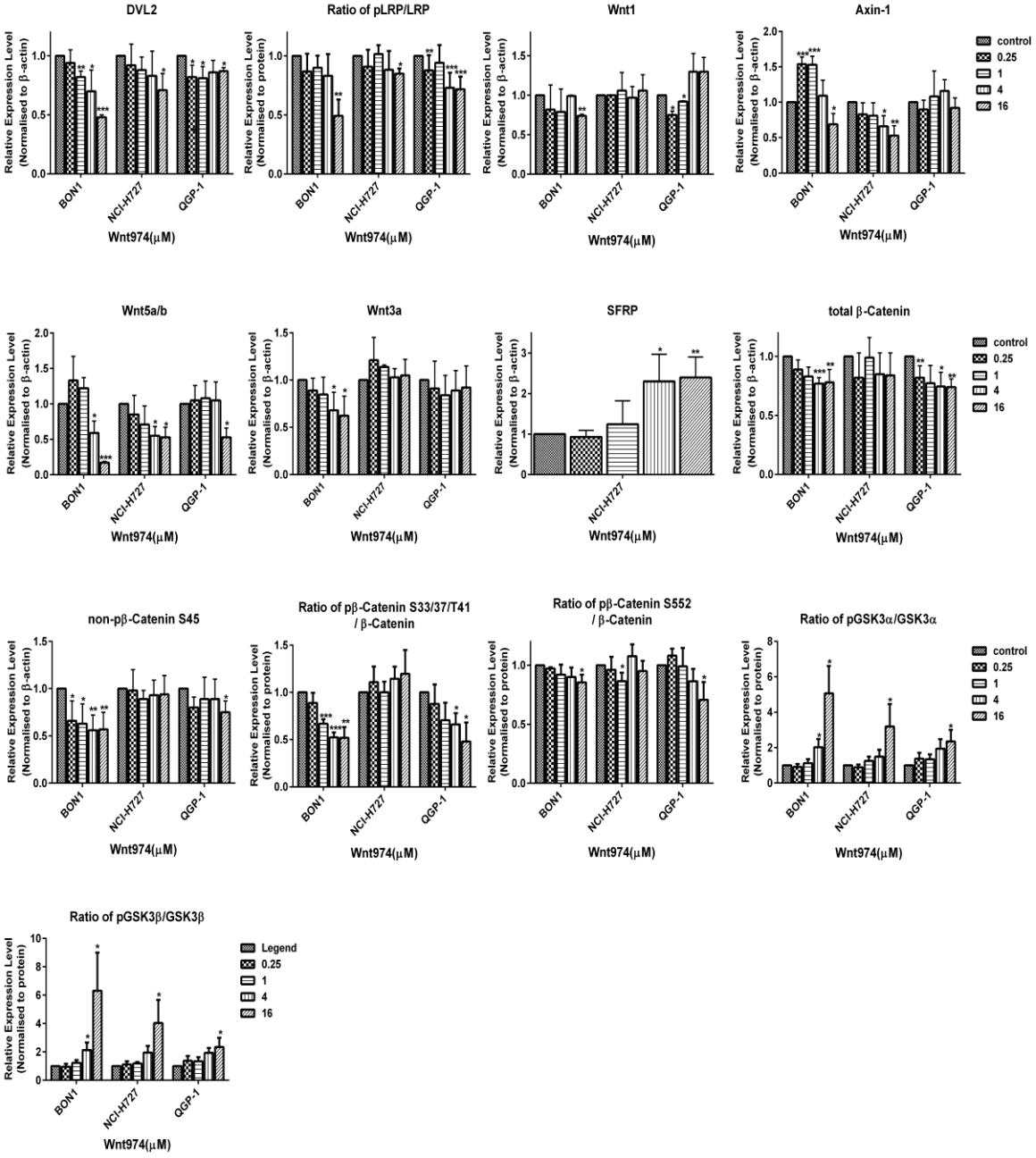

Fig.1-10. Quantitative western of suppressing wnt pathway after WNT974 treatment.

1.3.5 Inhibition of the pAKT/mTOR, pEGFR pIGFR and pJNK by WNT974

Since the modes of action of WNT974 on beta-catenin pathway were not unique in all three NET cells tested, we further assessed whether other prominent cell proliferation signaling could mediate the effects of WNT974. Our data showed that 48 h or 72 h treatment of NET cells with WNT974 had significant reduction of pAKT, S6, pS6, and p70S6K, whereas WNT974 was able to induce p-mTOR in these three cell lines (Fig. 1-11; Fig. 1-12).

WNT974 treatment also inhibited level of EGFR/pEGFR, and IGFR/pIGFR in Bon1 and QGP1 while reduced pERK in and H727 and QGP1. However, JNK/pJNK was inhibited only in Bon1 cells. These results further indicated that the inhibitory effects of WNT974 may be dependent on cell lines(Fig. 1-11; Fig. 1-12).

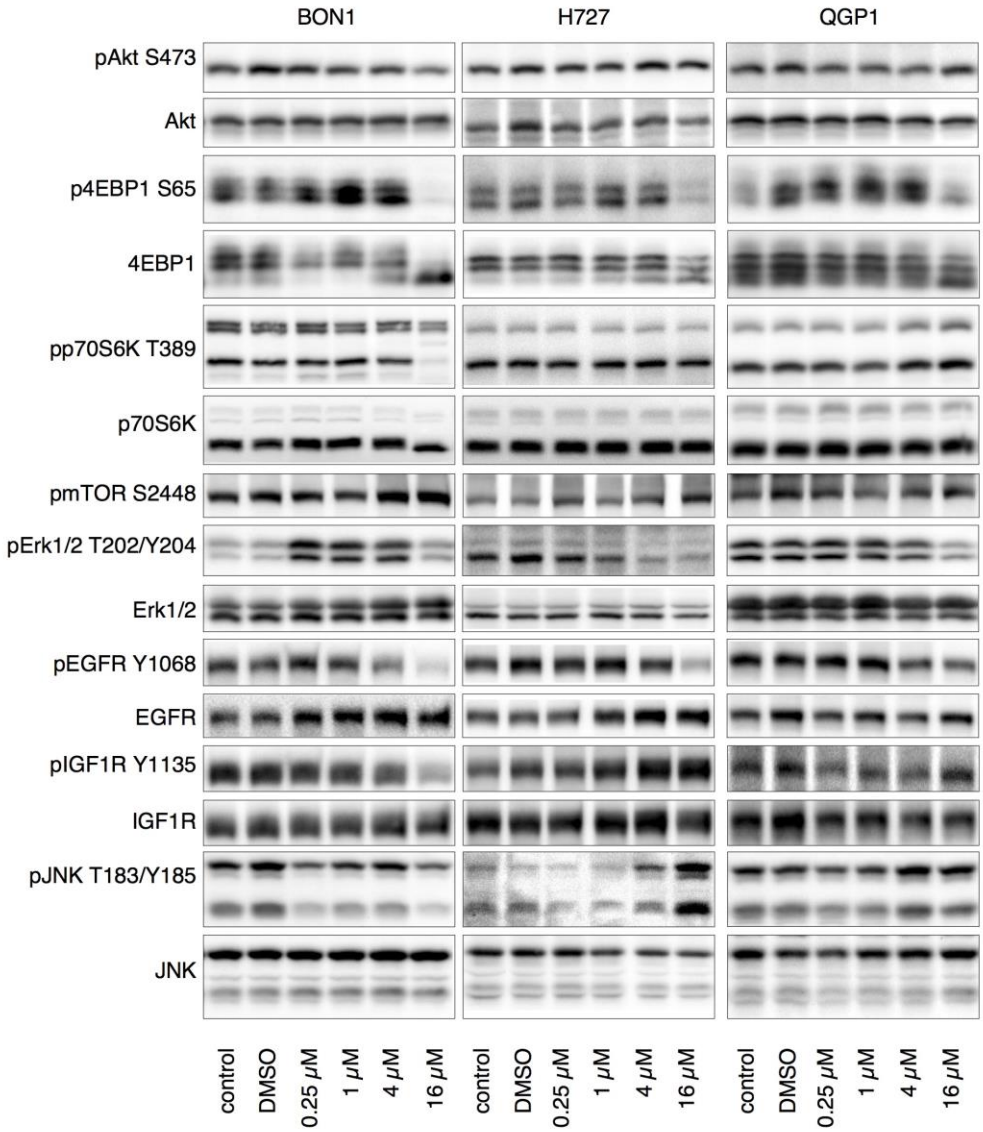

Fig.1-11. Effects of WNT974 on the inhibition of the pAKT/mTOR, MAPK/ERK, pEGFR and pIGFR pathways in NET cells.

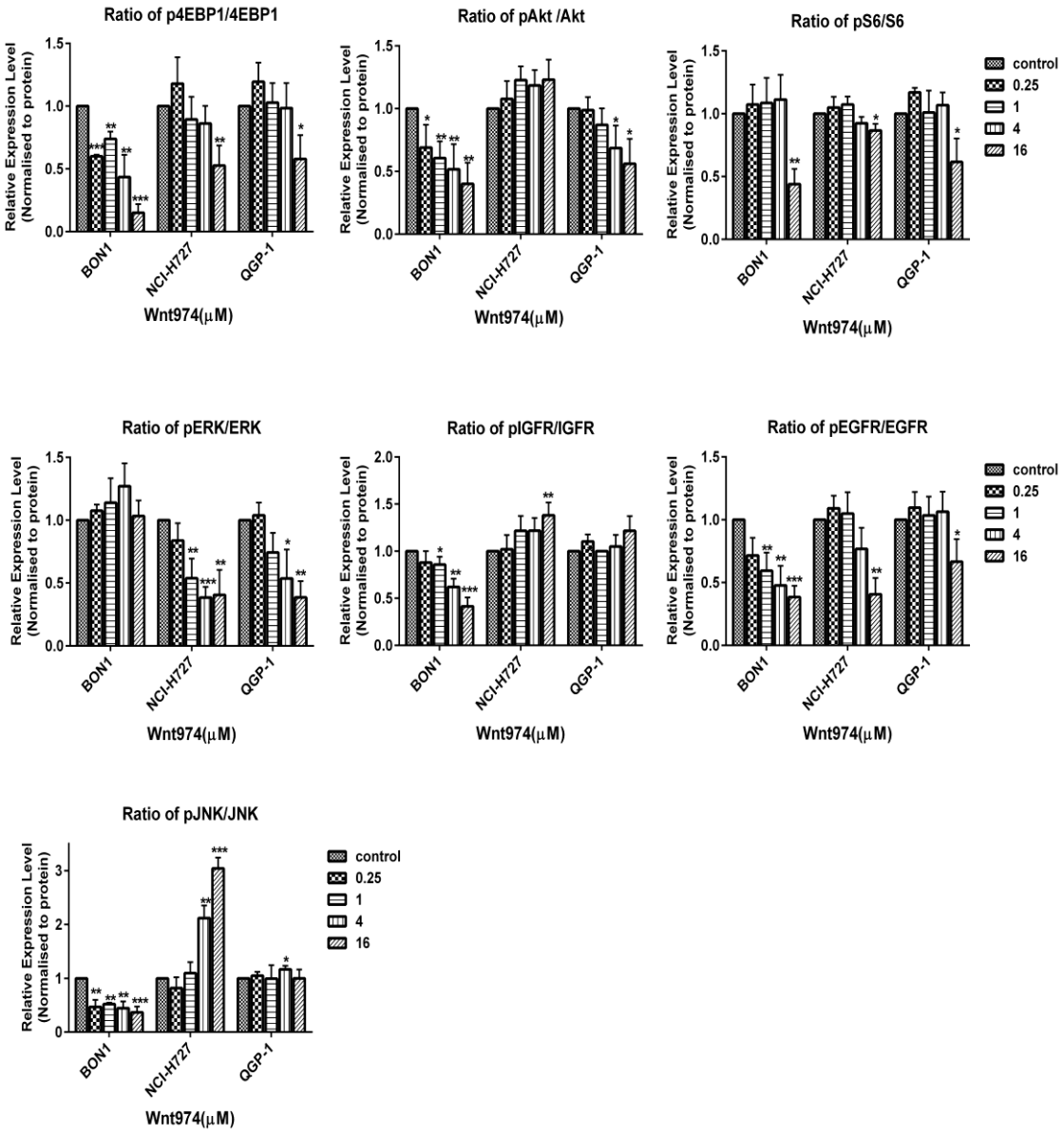

Fig.1-12.Representative quantification analysis of western blot of pAKT/mTOR, MAPK/ERK, pEGFR and pIGFR pathways post WNT974 treatment(*p, **p and ***p stand for <0.05, <0.01, p<0.001 respectively)**.**

1.3.6 The regulatory mechanisms of β-catenin siRNA and PRI724 (a molecule acting as a selective β-catenin inhibitor) on NET cells

We explored effect of β-catenin silencing and PRI724 on reduction of NET cell viability.Our data showed that β-catenin knockout markedly decreased the viability of Bon1 cells, but not that of H727 (Fig.1-13).At the protein levels, β-catenin siRNA also downregulated expression of CDK1, CHK1, DVL2 but upregulated pGSK3β (Fig.1-13). Combination of WNT974 with β-catenin siRNA could further suppress expression of these proteins, and also reduced cMyc and cyclinD1/cyclinD3, pLRP, pAKT, pERK1/2 and others.

As expected, PRI724, suppressed the NET cell growth significantly(Fig.1-14; Fig.1-15). PRI724 treatment also downregulated expression of non-p-β-catenin and β-catenin phosphorylation at S552 as well as cell cycle, and EMT-related proteins (cyclinD1, cyclinD3, CDK1/4/6, Chk1, cmyc and neurotensin) (Fig.1-14; Fig.1-15). Interestingly, PRI724 also downregulated expression levels of Wnt3a

but increased pGSK3beta (Fig.1-14; Fig.1-15). These data confirmed that PRI724 inhibited the NET cells in a similar way to that of WNT974.

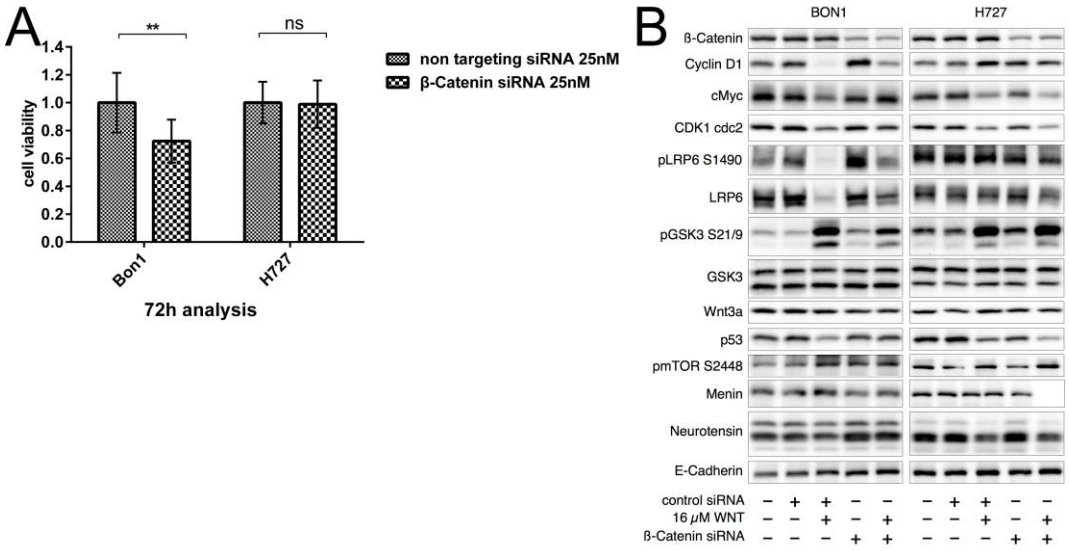

Fig. 1-13. **β-catenin siRNA regulated NET cell viability together with protein expressive levels.**

(Bon1 and H727 cells were transfected with β-catenin or negative control siRNA for 72 h and then subjected to the MTT assay and westernblot. *p, **p and ***p stand for <0.05, <0.01, p<0.001 respectively)

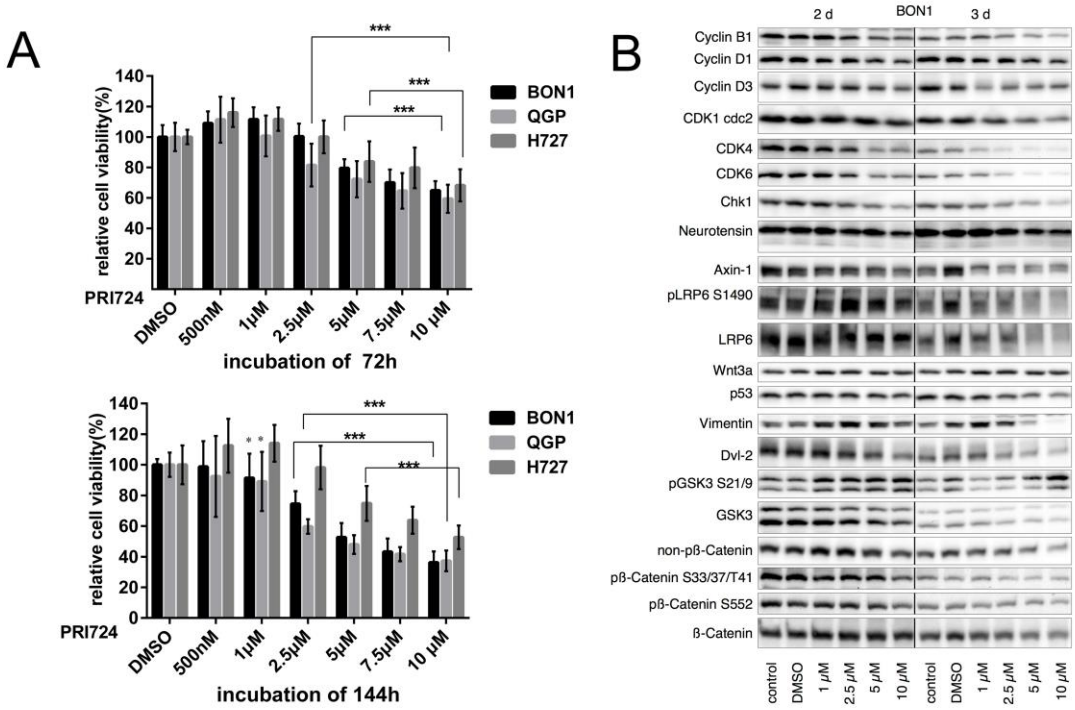

Fig. 1-14. Effect of PRI724, a selective β-catenin inhibitor, on the regulation of NET cell viability and protein expression.

(A, Cell viability MTT assay. B, Western blot. Bonl cells were treated with or without PRI724 (up to 10 μM) for 48h or 72 h and then subjected to A and B,*p<0.05, **p<0.01, and ***p<0.001 compared with that of controls.)

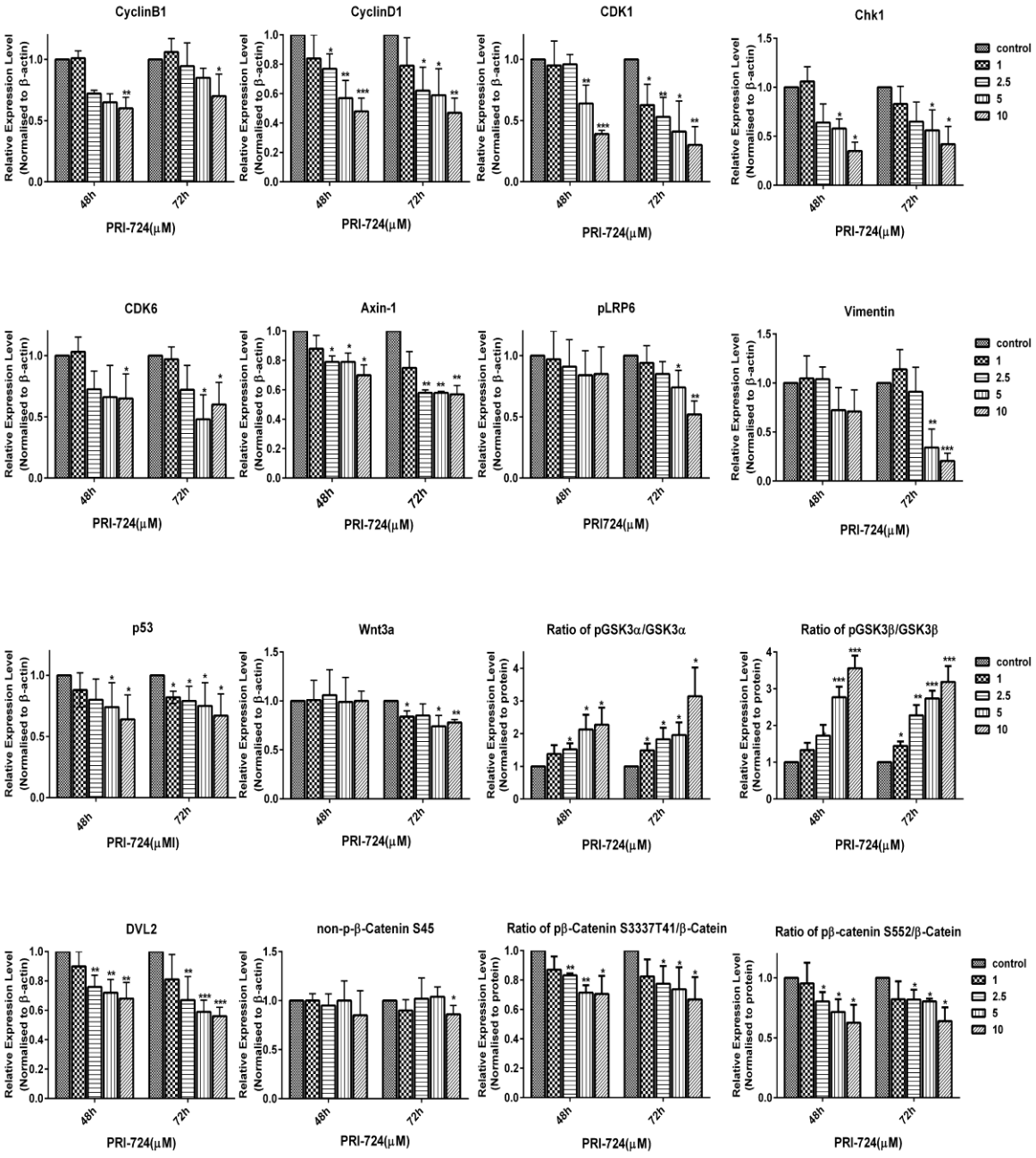

Fig. 1-15. Quantification of western blot analysis of PRI724 on inhibition Bon1 cells.

1.3.7 Effects of GSK3β siRNA with or without WNT974 on regulation of NETs

Next, we examined whether inhibition of GSK3β could enhance WNT974 suppression of NET cell viability. When the GSK3β siRNA was transfected to Bon1 or H727 , then approximately 80-90% GSK3β protein was knocked out, which led to a significant increase in NET cell viability (Fig.1-16). However, addition of WNT974 brought cell viability and p-GSK3β expression back to the control levels (Fig.1-16), indicating that WNT974 antitumor activity was through GSK3β inhibition. High expressions of p-GSK3β after WNT974 was detected in all the 3 cell lines, thus we postulated that the increase of p-GSK3β was cell dependent.

Indeed, GSK-3β siRNA transfection with or without WNT974 treatment significantly reduced β-catenin phosphorylation at Ser33/37/Thr41 and levels of ERK1/2/pERK1/2, AKT/pAKT, p70S6K, pJNK, pEGFR, and pIGFR (Fig.1-16). Knockdown of GSK3β expression also caused up-regulation pJNK in BON1 but

downregulation in H727. All in all, these further revealed that the suppression of WNT974 was in partially but not completely GSK3β-independent way.

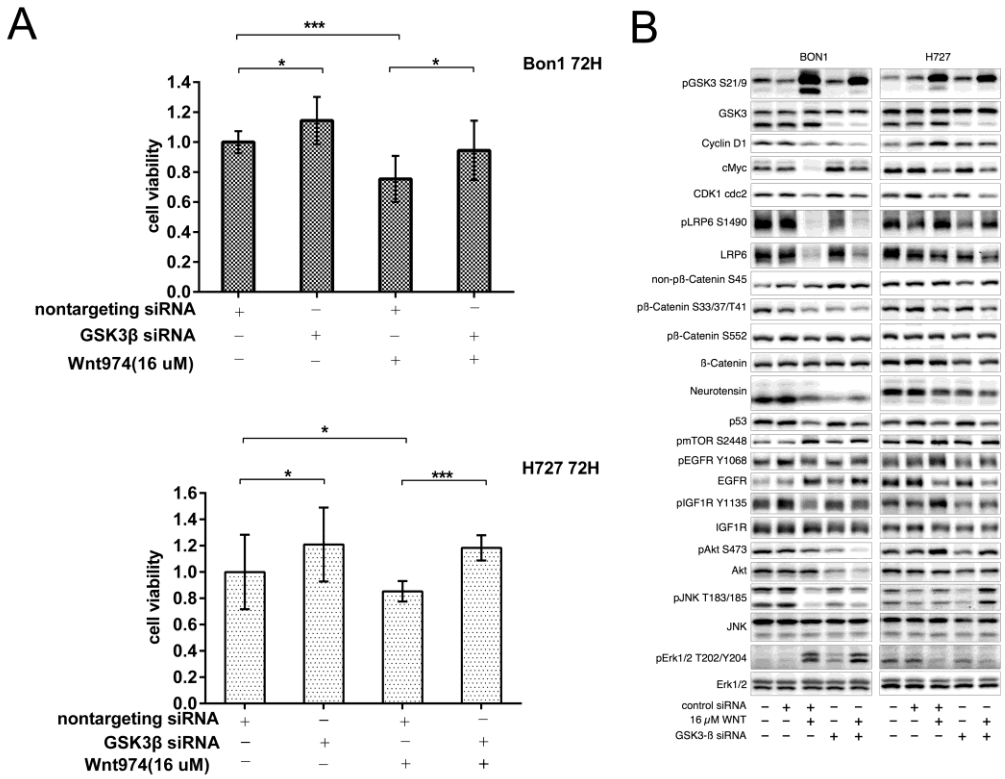

Fig. 1-16. GSK3 β siRNA regulated NET cell viability and protein expression.

(NET Bon1, and H727 cells transfected with GSK3β or negative control siRNA in presence or absence of WNT974 for 72 h and then subjected to A and B. A, Cell viability MTT assay . B, Western blot.)

1.3.8 The protein expression of neurotensin and menin was regulated by WNT974

Next, we explored whether WNT974 can regulate the expression of neuroendocrine tumor markers. Neurotensin (NT) has been reported to induce NET cell proliferation [80-81], Our western blot data showed that WNT974 and PRI724 both downregulated NT expression (Fig.1-8; Fig.1-14;1-15).

In addition, WNT974 slightly downregulated menin expression in all three NET cell lines, and after the knockout of β-catenin expression, menin was shown to be downregulated.Thus, we postulate that menin expression may be β-catenin-dependent. In contrast, we observed no constantly reproducible effects on Menin expression (Fig. 1-8; Fig.1-14;1-15).

1.4 Discussion

NETs are a group of diversified neoplasms with different malignant potential. Molecularly, NET demonstrate altered expressiion patterns of various genes and proteins, including Wnt/β-catenin, Ras/MAPK/ERK, Akt/mTOR, and Notch signal-

ing [9,82-83]. Indeed, our current study demonstrated that WNT974 concentration and time dependently suppressed the NET cell growth and lead to G1 and G2 phase arrest. At the molecular level, WNT974 treatment inhibited the Wnt/β-catenin, pAKT/mTOR, MAPK/ERK, pEGFR and pIGFR signaling pathways in NET cells. Furthermore, β-catenin siRNA reduced NET cell viability and down-regulated the expression of cyclinD1, CDK1, but upregulated the expression of pGSK3β.

PRI724 treatment also downregulated the expression of non-p-β-catenin at Ser45, phosphorylated β-catenin at S552 and Ser33/37/T41 as well as cell proliferation, cell cycle, and EMT-related markers. In contrast, the knockdown GSK3β expression significantly increased NET cell viability, whereas the addition of WNT974 treatment antagonized the effect of GSK3β siRNA on cell viability and p-GSK3β expression.Altogether,our current study demonstrated that targeting Wnt/β-catenin signaling could potently induce antitumor effects against NET cells, although there was no apoptosis involved in WNT974-treated NET cells in vitro.

Recent studies showed that Wnt signaling activation or inhibition depended on receptor context or distinct receptors in different cell types [69,70]. We revealed that the expression of pLRP6, pDVL2, and wnt3a/5ab was downregulated by WNT974 in all three cell lines, which was indicative that the Wnt canonical and non-canocical pathways were important in NET cells. The Axin1 protein expression initially rised and then gradually decreased after consistent WNT974 treatment. This biphasic process might be because i) Axin crosstalks with many other signaling pathways(e.g. TGF-β/Smad3 cascade[84]; ii) Axin is a concentration-limiting factor in the β-catenin degradation complex[85-86]; and iii) The expression of Axin1 was cell type-dependent [84,85-86]. In our current study, we demonstrated that WNT974 decreased the expression of non-phosphorylated β-catenin, total β-catenin, which primarily resulted from an increase in the proteasome-mediated degradation of β-catenin. Furthermore, our current study showed that with the exception of wnt canonical signaling, the pAKT/mTOR, pERK, pEGFR and pIGFR signaling pathways were also downregulated by WNT974 in

NET cells, indicating that the dual or multiple suppression of different signaling pathways could be more feasible and efficient to control NET.

In the study, WNT974 caused G0/G1 arrest in Bon1 and H727，whereas a G2 phase arrest was observed in QGP, demonstrating the heterogeneity of different types of NETs. Further analysis showed that WNT974 downregulated cyclinD1/cyclin D3, CDK1/2/4, chk1/2.

Meanwhile in all the 3 cell lines no concurrent upregulation of apoptosis was observed. All these findings suggest that WNT974 should be further evaluated in combination with chemotherapy, radiotherapy and other molecular targeting inhibitors to further enhance its anti-tumor effects.

GSK3β could function as oncogene as well as tumor suppressor in various cancers. In NETs we have proved previously that decreased active GSK3 or increased inactivated GSK3beta was indicative of antitumor effect in NETs [87,88-90].

Other previous studies revealed that GSK3 was essential in wnt/beta catenin cascade, although only a small percentage (<10%) of the total GSK-3 in cells is to engage in the canonical Wnt signaling[91-92]. The presence of phosphorylated-GSK3β(Ser9) means the deactivation of GSK3β; however, the overexpressive p-GSK3β does't mean loss of GSK3β, and an increase of p-GSK3β was cell-dependent(as indicative of the aspirin treated colorectal cancer cells) [93,94,95].

Our current study showed that GSK-3β silencing enhanced cell viability of Bon1 and H727 and that WNT974 treatment could block the impact of GSK-3β knockout on NET cell growth and protein expressions, indicating that the WNT974-reduced NET cell growth occurred through the inhibition of GSK3β signaling. Moreover, our current data showed that GSK-3β knockdown downregulated the level of phosphorylated β-catenin, cyclinD1, CDK1, neurotensin, pLRP (Fig.1-16); however, the co-treatment of these cells with WNT974 further suppressed the expression of these markers. In addition, GSK-3β siRNA reduced the expression pEGFR, pAKT,

pERK, pJNK but upregulated the expression of c-Myc and p-mTOR (Fig.1-16). Altogether, the downregulation of AKT/pAKT, ERk/pERK,JNK/pJNK, IGFR/pIGFR and EGFR/pEGFR by WNT974 was GSK3 independent as well as cell-line dependent.

The reasons for this discrepancy remain to be clarified and might be discussed as follows:

i) a counter-regulatory mechanism or negative feedback effects as a result of inhibition of Wnt canonical signaling might lead to downregulation of GSK3 and restoration of beta catenin signaling.

ii) the increase of p-GSK3β might be cell- and tissue- context dependent.

iii） GSK3 is envolved in various signaling pathways and crosstalks with many other signaling pathways.

iv) GSK3α and GSK3β are not inter changeable ,which are both necessary for the entire function of GSK3.

Menin seems to be a negative regulator of beta-catenin, and in MEN1-deficient

knockout mice beta-catenin is activated in pNETs while vice versa conditional beta-catenin knock-out decreased tumourigenesis of pNETs[40,96] .More clinical studies are essential to investigate the precise interaction between menin and beta-catenin in NET.

Our current study primarily sought to assess the antitumor activity of WNT974 in NET cells in vitro. Although we also provided the underlying molecular events triggered by this inhibitor in NET cells, the major limitations of our current study are that i) We did not monitor the Wnt-regulated transcriptional activity using a luciferase reporter assay; ii) We did not knock down each of these key gene pathway genes to assess the precise targets of WNT974 and PRI724 action; and iii) We did not assess the combination of these two agents or their combination with other chemotherapeutic drugs on NET cells.

In conclusion, our current study provides information on the molecular events of WNT974 and PRI724 anti-cancer activities in NET cells in vitro. Our current observations lead to two possible conclusions, i.e.,

i) WNT974 inhibits Wnt/β catenin signaling and

ii) WNT974 exerts an inhibitory effect on NET cells by downregulating the MAPK/ERK, PI3K/AKT/mTOR, pEGFR, and pIGFR non-canonical signaling cascades.

However, the context-dependent effects of WNT974 on NET need further investigation.

2 Chapter two: Combination of 5-fluorouracil (5-FU) with DNMT inhibitors or HDAC inhibitors as epigenetic modifiers has synergistic effects on radiosensitization and somatostatin receptor 2 (sstr 2) expression in human neuroendocrine tumor cells

2.1 Introduction

Treatment of NETs, includes surgical resection, if diagnosed at the early stage of disease, while advanced disease requires systemic treatment strategies as biotherapy, chemotherapy, targeted therapy and peptide receptor radionuclide therapy(PRRT) [97-98,26].

Peptide receptor radionuclide therapy (PRRT) uses radiopharmaceuticals that target peptide receptors e.g. somatostatin receptor type 2 (SSTR2) expressed on the tumor cell surface to deliver specific binding of the radiopharmaceutical and radiotherapy to NETs [99-101]. To date, [177 Lu-DOTA0, Tyr3]-octreotate

(177Lu-DOTA-TATE) is the most frequently used radiopharmaceutical to be used for PRRT in NETs [99-101].

PRRT in patients with NETs causes an objective response rate (ORR) less than 30%, while the disease control rate (DCR) is much higher due to common stable disease following PRRT[99-101]. PRRT significantly improves OS and PFS in NETs and has limited side effects[99-101] .

Complete remission of NETs following PRRT with [177Lu-DOTA0, Tyr3]-octreotate (177Lu-DOTA-TATE) is a rare event in 2-7% and also partial remissions occur only in 19 -29 % of patients [99-102]; optimized treatment strategies are imperative to further improve PRRT efficacies in NETs. An essential requirement for PRRT of NETs is the abundance of the SSTR receptors expressed in NET cells[100-101], although the predictive value of somatostatin receptor expression measured as SUV in a pretherapeutic PET/CT seems modest and variable [[100-104].

Several clinical studies have demonstrated that PRCRT using the combination of chemotherapy with 5-fluorouracil[26], capecitabine[105], or capecita-

bin/temozolomide [106], plus PRRT could offer highly efficient objective tumor response and effective tumor control of NETs [101,105-106]. These effects of PRCRT were suggested to be mainly due to radiosensitizing effects[101], while radionuclide uptake during PRRT has been reported to be not significantly altered by concomitant chemotherapy with capecitabin /temozolomide[27].

Furthermore, DNA methylation/demethylation and histone acetylation/deacetylation are fundamental factors in genetic modifacations[27]. DNA methyltransferase(DNMT) transfers methyl groups to genomic DNA, usually at CpG islands, to methylate DNA and silence gene transcription [107]. Moreover, the HDAC transfers acetyl groups to induce histone to tightly bind to genomic DNA and therefore, acetylation and de-acetylation will regulate gene expression[108-109]. HDACi or DNMTi has been established to treat various human cancers [31-32]. Combination of HDACi with DNMTi, conventional cytotoxic chemotherapy and radiotherapy has been investigated in various cancers and has been suggested to be additive or synergistic [33].

In neuroendocrine tumors, epigenetic alterations and epigenetic modulation by HDAC inhibitors（HDACi） and DNMT inhibitors(DNMTi) as potential treatment strategy have been studied and reviewed recently [44,110-113]. Preclinical studies in NETs demonstrated that many DNMT inhibitors e.g. decitabin (DEC) and HDAC inhibitors eg. valproic acid (VPA) or tacedinalin (TAC) could induce the expression of somatostatin receptor type 2 (SSTR2) [114-115] and promote the binding of somatostatin ligands to the targeted tumor cells [114-115]. DEC and TAC were demonstraed to be the most potent drugs investigated and their combination also demonstrated synergistc effects [114].

Therefore, we explored the underlying molecular basis of 5-fluorouracil (5-FU) in combination with or without DEC or TAC on cytotoxicity, apoptosisis, cell cycle changes, somatostatin receptor type 2(SSTR2) expression and somatostatin ligand binding of 68Ga-DOTA-TOC in various NET cell lines in vitro. Radiosensitizing effects as well as increased somatostatin receptor expression were demonstrated for each compound tested and synergistic effects were found combining 5-FU and

epigenetic modifiers. Thus, these preclinical in vitro data suggest to further investigate the possible role of 5-fluoropyrimidine based chemotherapy in combination with epigenetic modifiers and PRRT as a novel strategy in peptide receptor chemoradiotherapy (PRCRT) in NETs.

2.2 Material and Techniques

2.2.1 Cell lines, reagents and cytotoxicity assay

The cell culture methods used were similar to those in chapter 1 (please refer to chapter one). 5-FU, DEC, and TAC were all obtained from Selleckchem (Munich, Germany) and stock solution of these agents were prepared by dissolving them in dimethyl sulfoxide (DMSO). ^{68}Ga-DOTA-TOC was produced by using SomaKit TOC^{TM} , which was ordered from AAA Inc (Saint-Genis-Pouilly, France. The ^{68}Ga was eluted from a GalliaPharm® Ge-68/Ga-68 generator from Eckert & Ziegler (Berlin, Germany).

To assess the effects of DEC or TAC on enhancement of 5-FU anti-tumor activity, NET cells were treated with these two drugs concurrently at a fixed ratio of ap-

proximating their individual IC20 (drug dose that reduce 20% of cell viability on day 6) for 144 h and the doses of 5-FU were selected based on our previous studies [116]. Cell Titer Blue was utilized for testing cytotoxicity as described in chapter one .

2.2.2 Tumor cell colony-forming analysis

(1) Bon1 and QGP tumor cells were plated into six-well plates.

(2) Cells were grown and exposed to low concentrations (approximately IC10 or IC20) of 5-FU, DEC, TAC, 5-FU+DEC, 5-FU+TAC,and DEC+TAC for 24 h.

(3) Cells were exposed to various doses (0Gy, 1Gy, 2Gy, 4Gy, 6Gy) by using a XStrahl RS225 radiation cabinet (XStrahl Inc., Camberley, UK).

(4) Cells were further incubated for 2 weeks (Bon1 cells) or 3 weeks (QGP1 cells) .

(5) Afterwards NET cells were fixed with 70% ethanol and then stained with 0.3% methylene blue for 0.5-1 h.

(6) Then the colonies (greater than 50 cells) were counted and the surviving fractions (SF) were calculated vs. the plating efficiency.

(7) Linear-quadratic (LQ) models were used to fit the survival curves, the sensitization enhancement ratio(SER) was determined as followings:SF= $1-(1-\exp[-D/D_0])^N$;SER_{SF2}= the SF2 value of DMSO control group /the SF2 of various treatment group [117-120] .

2.2.3 γH2AX/53BP1 immunofluorescent staining after γ-irradiation

First, we seeded BON1 into 24-well plates with the number of 40.000 cells per well, then the second day, we administrated the cells with DEC (0.5 μM) or 5-FU (1.0 μM), we also used DMSO as negative control. After 24 h, we discard the drugs and changed new freshed medium, and exposed the NET cells to gamma irradiation at a dose of 2 Gy. Next, we used 3.7% isotonic formaldehyde to fix NET cells at various time points (0 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h) and stained them for 53BP1/γhistone H2AX (γH2AX, p S139) .We captured 31 z-stacks images with an inter-stack interval of 250 nm by immunofluorescence microscopy(ZEISS Observer Z1 ,Carl Zeiss, Germany). Then we utilized the ZEN software(Carl Zeiss, Ver-

sion 2.3) to perform the deconvolution. We counted the numbers of γH2AX foci of 20 nuclei manually, and calculated the means ± s.d for the different treatment groups at all time points.

2.2.4 Protein isolation and Western blotting

The westernblotting assay was performed as described in chapter1 please refer to part 1). We tested the following antibodies: anti-SSTR2 (Abcam), PARP, cleaved PARP, caspase-3, cleaved caspase-3(Cell Signaling), or CDK1/CDK4/CyclinD1/ CyclinB1/Chk1 /p53 /Bcl-2 /p21Waf1/Cip1(see Table.1- 2,Chapter one) .

2.2.5 Cell cycle assay by flow cytometry

The flow cytometry analysis was performed as described in chapter one (please refer to part 1).

2.2.6 ^{68}Ga-DOTATOC uptake assay

The BON1 and H727 cells were treated with 5FU+DEC, or 5FU+TAC for 72h (using the same concentration as used in the westernblot experiments), then the supernatant was removed and cells were further cultured with the internalization

medium (DMEM) for 1 h, and then we added with ^{68}Ga-DOTA-TOC-labeled peptides (4 kBq) in a final peptide concentration of 2.5 pmol/L, then further cultured the cells to indicated time points (0.5, 2, and 4 h) at 37°C. Excessive non-labeled ^{68}Ga-DOTO-TATE (1000 fold) was used as the nonspecific internalization control. The cells were then exposed to 1M NaOH at 37°C for 10 minutes at least 3 times to remove the cells from the dishes. The gamma ray-counter (PerkinElmer) was used to measure the radioactivity, which was expressed as the ratio of measured to total amount of radioactivity and normalized to the total protein levels.

2.2.7 Statistical analysis

Statistical analysis was performed as described in chapter1 please refer to chapter one). CalcuSyn 2.1 (Biosoft, USA) [121-123] were used to evaluate the synergistic additive or antagonistic effects of the 5FU combined with DEC or TAC on NET cells.

2.3 Results

2.3.1 Dose-dependent reduction of NET cell proliferation after in vitro treatment with DEC or TAC

In this study, we first treated different NET cell lines with DEC and TAC and found that these two drugs were able to individually reduce NET cell growth at a dose-dependent fashion. After 144 h treatment, cells treated with TAC showed a median viability of 11%, 35.9%, and 42.04% for Bon1, QGP, and H727 cells, respectively (Fig.2-1), while cells treated with decitabine showed a median viability of 49%, 88%, and 84% in Bon1, QGP, and H727 cells, respectively. The IC50 of DEC and TAC in all these 3 cell lines is shown in Table 2-1.

NET cell lines	**IC50**	
	Decitabine(μM)	Tacedinaline(μM)
Bon1	15.19	3.58
QGP	—	11.97
H727	—	15.68

Table 2-1. IC50 of Decitabine and Tacedinaline in NET cells.

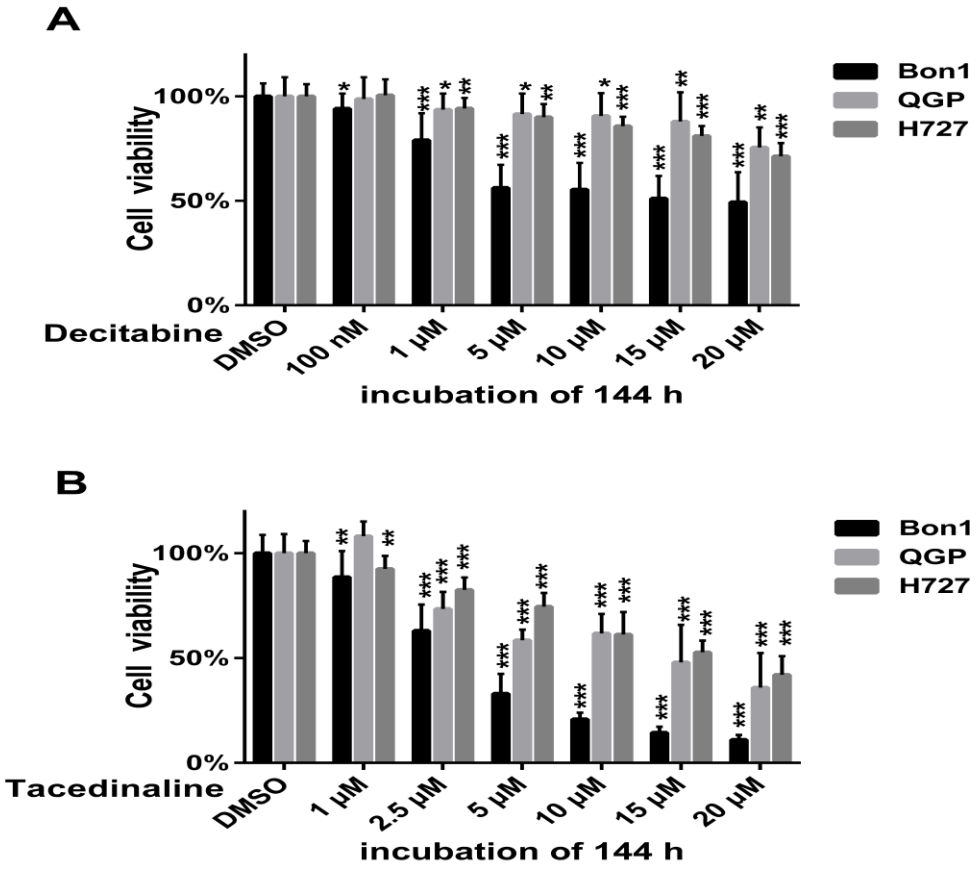

Fig. 2-1. DEC and TAC decreased NET cell viability.

2.3.2 5-FU plus DEC or TAC exerted synergistic effects on cell proliferation in vitro

Next, we assessed the combination therapy of 5-FU with DEC or TAC on NET cell proliferation in vitro. Not only 5-FU plus DEC but also 5-FU plus TAC caused syn-

ergistic antiproliferative effects in all cell lines included (Fig.2-2). The combination index (CI), calculated as the fractional cell growth inhibition (FA) as a function of the CI, was less than 1 in the combination of 5FU with DEC [the mean CI (the FA50) was 0.323, 0.80, and 0.859, respectively for these three cell lines], indicating that the treatment with two different drugs indeed showed the synergistic effects on these NET cells. The combination of 5-FU with TAC could reach the CI of 0.556, 0.782, and 0.70, respectively in Bon1, QGP, and H727 cells (Fig.2-2 and Table.2-2).

Cell lines	Combination therapy	ED50	ED75	ED90
Bon1	5FU+DEC	0.32	0.20	0.16
	5Fu+TAC	0.55	0.53	0.62
QGP	5FU+DEC	0.80	0.84	0.88
	5FU+TAC	0.78	0.91	1.03
H727	5FU+DEC	0.85	0.70	0.58
	5FU +TAC	0.70	0.55	0.48

Table 2-2. Combination index(CI) values of 5-FU plus DEC or TAC in NET cells.

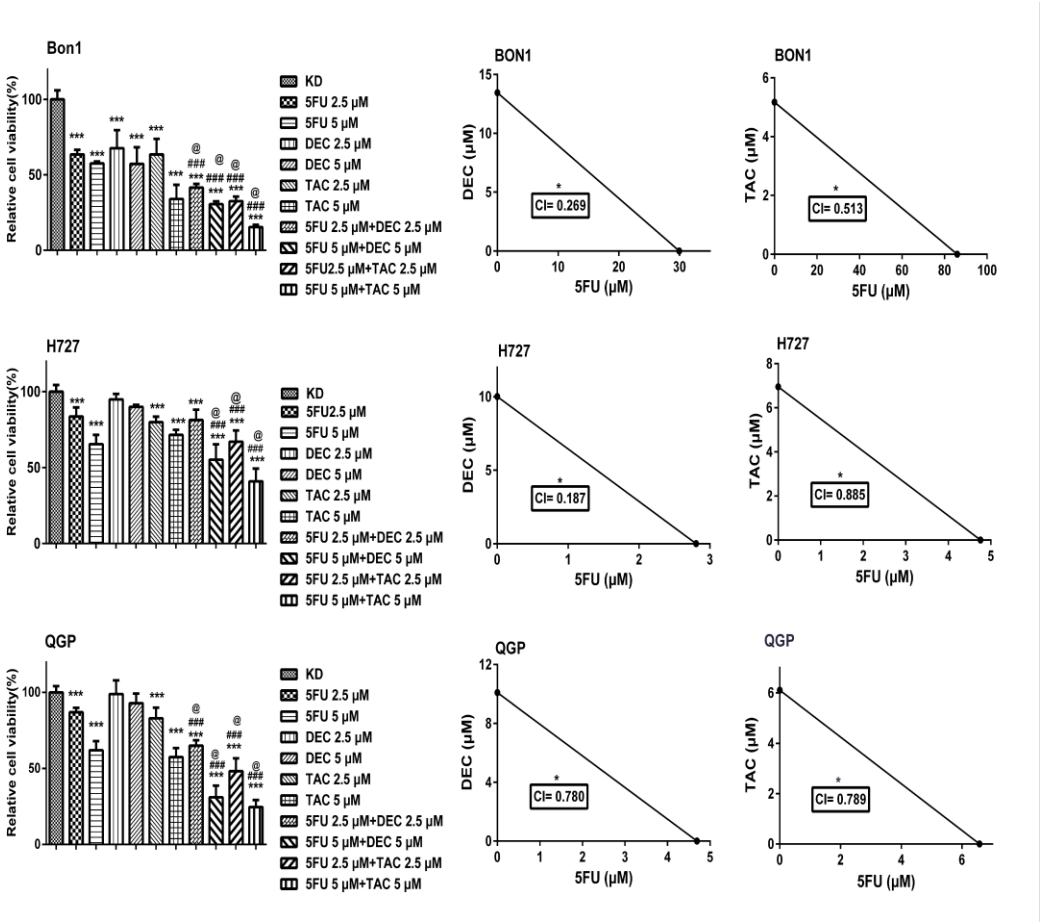

Fig. 2-2. 5-FU combination with DEC or TAC synergistically suppressed NET cell viability.

(The isobologram plots and combination index were calculated based on different combination treatments.)

2.3.3 5-FU combined with DEC or TAC synergistically inhibited the clonogenic survival after γ-irradiation in NET cells

We next explored whether the combinations of 5-FU with DEC or 5FU with TAC had synergistic effects on radiosensitizing NET cells.

Therefore, we treated Bon1 and QGP1 cells with low concentrations of 5-FU (1 μM) with or without DEC or TAC at doses of 0.5 μM for DEC and 2.5 μM for TAC (approximately IC10 to IC20 dosage) and subjected them to clonogenic assay. The low dose of 5-FU (1 μM) was based on our previous studies[116] and preliminary work performed in our laboratory confirmed its radiosensitizing effect.

The sensitization enhancement ratios (SERs) for 5-FU, DEC, TAC, 5FU + DEC , 5FU + TAC and DEC + TAC，in Bon1 cells were achieved to be 2.19, 1.10, 1.10, 1.27, 3.96, 9.46, and 1.10, respectively, and 1.20, 1.12, 1.01, 1.31, 1.87, and 2.42 respectively, in QGP1 cells.

Thus, 5-FU plus TAC treatment was the most effective approach in reducing the clonogenic survival in BON1 cells with superiority to combinations of 5-FU and

DEC, or DEC and TAC, or each single drug (Fig.2-3). In contrast, DEC plus TAC was most effective in QGP1 cells, followed by 5-FU plus DEC and 5-FU plus TAC vs. each single agents (Fig.2-3)

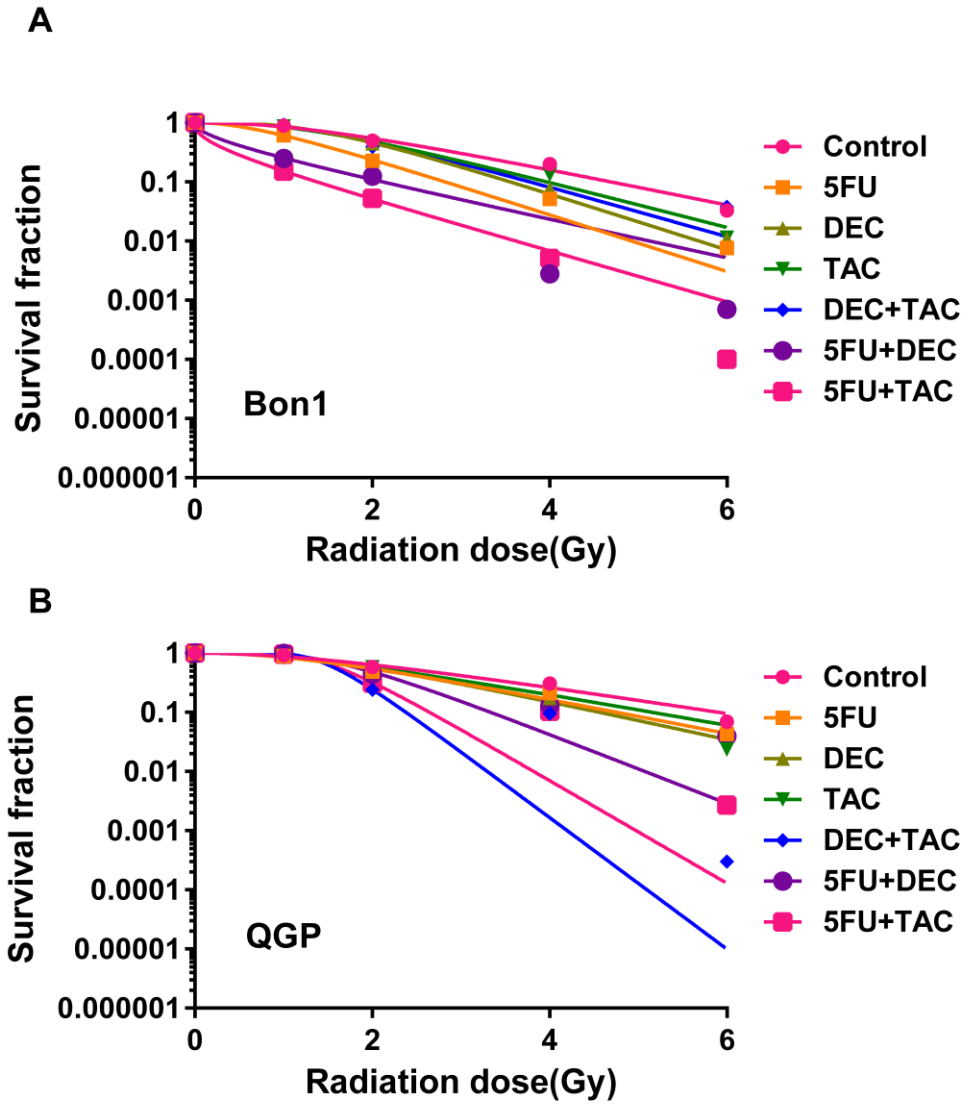

Fig. 2-3. 5-FU plus DEC or TAC reduced cell colonic formation of NETs.

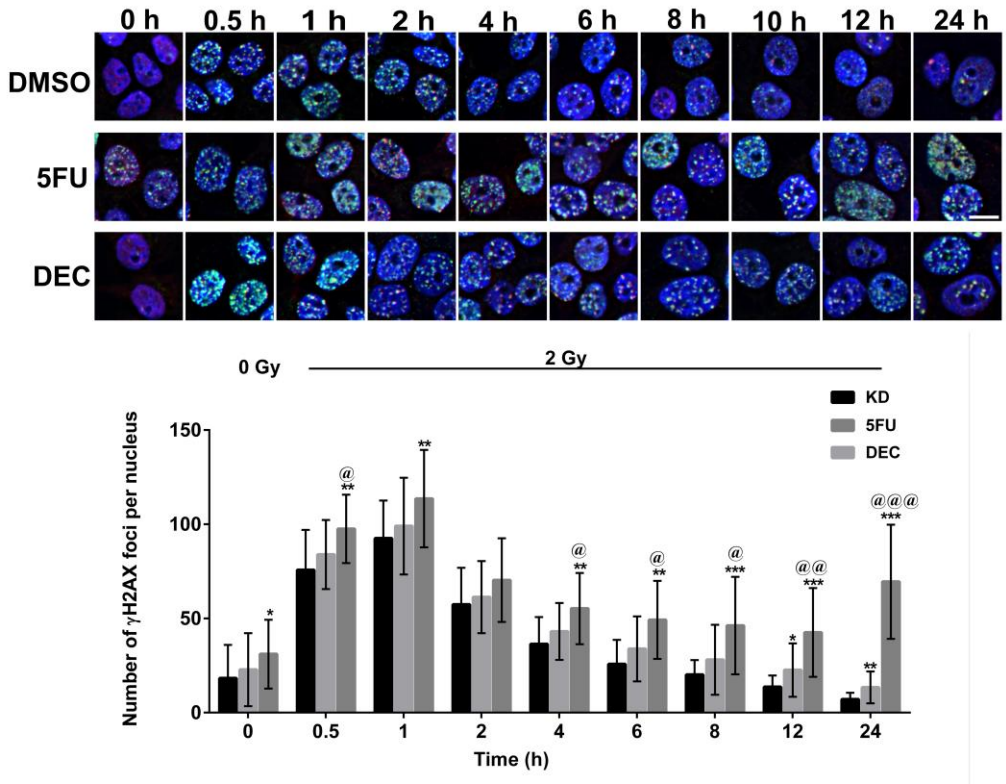

Fig. 2-4 5FU and DEC caused persistent immunofluorescence staining of γH2AX/53BP1. (A)representative picture of phospho-H2AX and 53BP1 immuno-fluorescence staining after irradiation. Green stands for phospho-H2AX; Red stands for 53BP1; Blue stands for Hoechst 33342 (2ug/ml). (B) the clearance of γ-H2AX foci.*p<0.05, **p<0.01, and ***p<0.001(5FU/DEC vs DMSO control) .$^{@}$ p<0.05(5FU vs DEC).

2.3.4 5-FU and DEC reduced the efficiency of DNA damage repair after γ-irradiation

The combined administration of 5-FU or decitabine with γ-irradiation significantly suppressed efficiency of DNA damage repair in BON1 cells.

Both, 5FU or DEC caused an increased persistence of γH2AX-/53BP1 DNA damage foci even 24h following the irradation($p<0.001$), which indicated that both drugs decreased the efficacy of DNA damage repair. 5FU was significantly superior to DEC in displaying DNA damage response foci at 4/6/8/12/24 h respectively ($p<0.01$, Fig. 2-4).

2.3.5 Effects of 5-FU combination with DEC or TAC on NET cell cycle re-distribution and gene expression

In Bon1 cells, 5-FU or 5-FU plus DEC treatment induced a significant rise of G2 phase and a subsequent reduction of G1 and S phase, while 5-FU combination with TAC caused tumor cell arrest at the G1 cell cycle (Fig.2-5). Similar findings also occurred in H727 cells but not in QGP1, i.e., 5FU plus DEC caused accumu lation of the G1/M phased cells (Fig.2-5), whereas treatment of cells with 5-FU and TAC did not result in significant changes in the proportion of the G1 and S phases.

These data indicate that the differential effects of these drug combinations on cell cycle distribution could be cell -type-dependent.

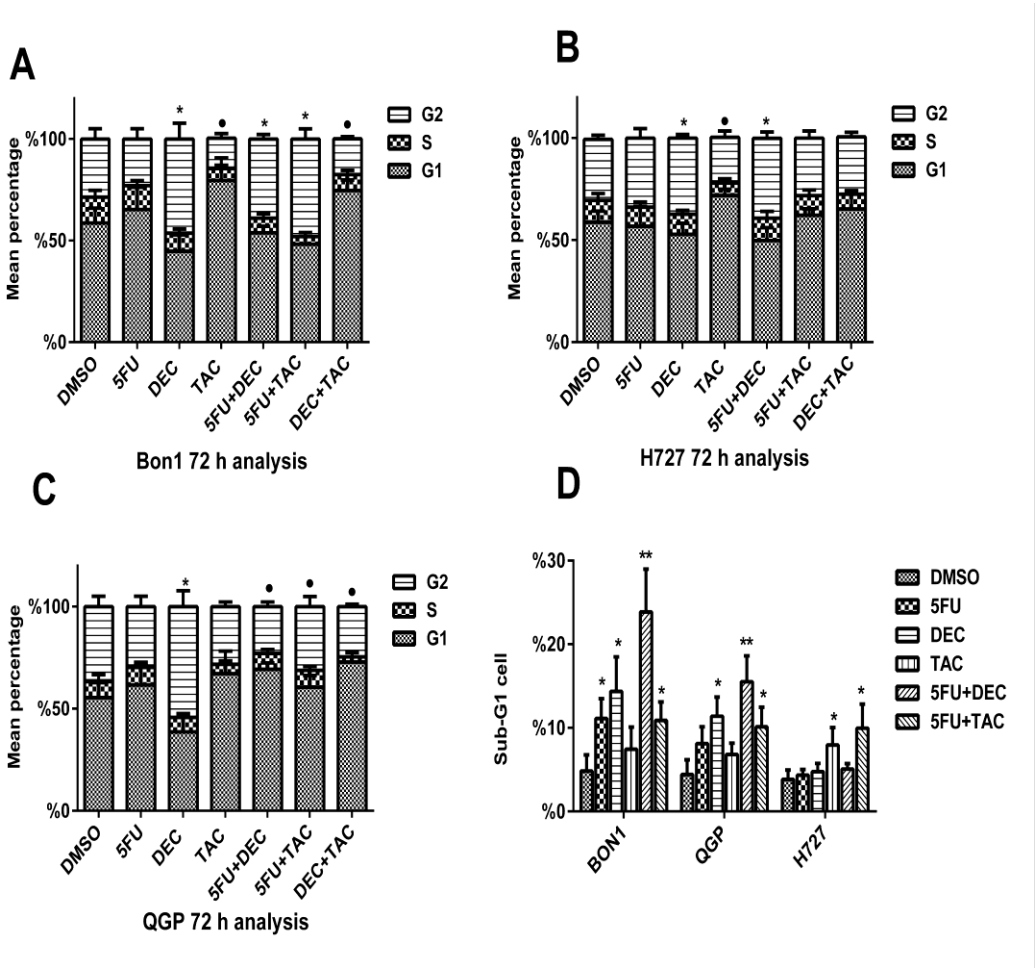

Fig. 2-5. Effects of 5-FU combination with DEC or TAC on NET cell cycle re-distribution (*p, **p and ***p stand for <0.05, <0.01, p<0.001 respectively).

In radiation therapy, the G2/M phased cells are the most radiosensitive [124]; thus, treatment of NET cells with 5-FU plus DEC or TAC could sensitize NET cells to

radiation therapy. At the protein level, 5-FU with TAC decreased expression of Chk1, CDK4, and CyclinD1 more significantly compared to the control group or each single agent (Fig.2-6). In contrast, pretreatment 5FU with DEC maintained the high levels of Chk1 and CDK4 expressions, although slightly decreased cyclinD1, CDK6 expression (Fig.2-6 , Fig.2-7)

2.3.6 Effects of 5-FU combination with DEC or TAC on NET cell expression of apoptosis related proteins

Western blot data showed that incubation of Bon1 cells with 5-FU, DEC, 5-FU + DEC, TAC, or 5-FU + TAC induced expression of cleaved-PARP 2.8-, 3.2-, 4.4-, 0.7-, and 1.4-fold , indicating 5-FU plus DEC being much stronger than either single drug (Fig.2-6,Fig.2-7). Moreover, in H727 cells, expression of the cleaved PARP was increased by 1.17 (5-FU alone), 1.55 (DEC alone), 1.89 (5-FU + DEC), 1.25 (TAC alone), 2.34 (5-FU + TAC) folds, respectively.

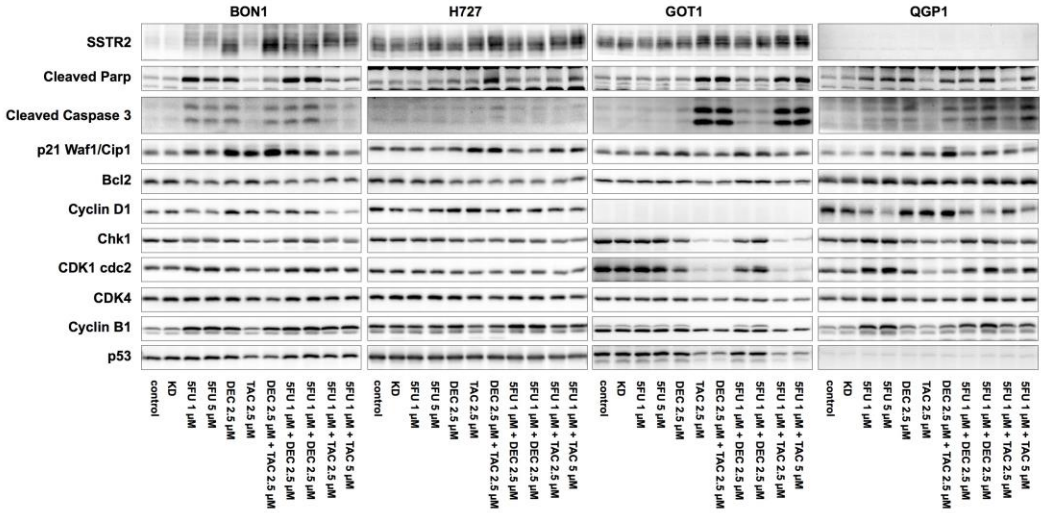

Fig. 2-6. Effects of 5-FU combination with DEC or TAC on NET cell expression of SSTR2, apoptosis-related protein and cell cycle-related protein.

The cleaved caspase 3 level was slightly upregulated in BON1, QGP as well as H727 cells ,while it significantly increased in GOT1 cells after 5-FU + DEC/5-FU + TAC treatment(Fig.2-6, Fig.2-7). In accordance, expression of the anti-apoptotoc protein Bcl-2 was reduced to 0.63 (5-FU), 0.75 (DEC), 0.61 (5-FU + DEC), 0.84 (TAC), and 0.70 (5-FU + TAC), respectively in Bon1 cells and by 1.0, 0.83, 0.68, 0.71, and 0.72, respectively in H727 cells (Fig.2-6, Fig.2-7).

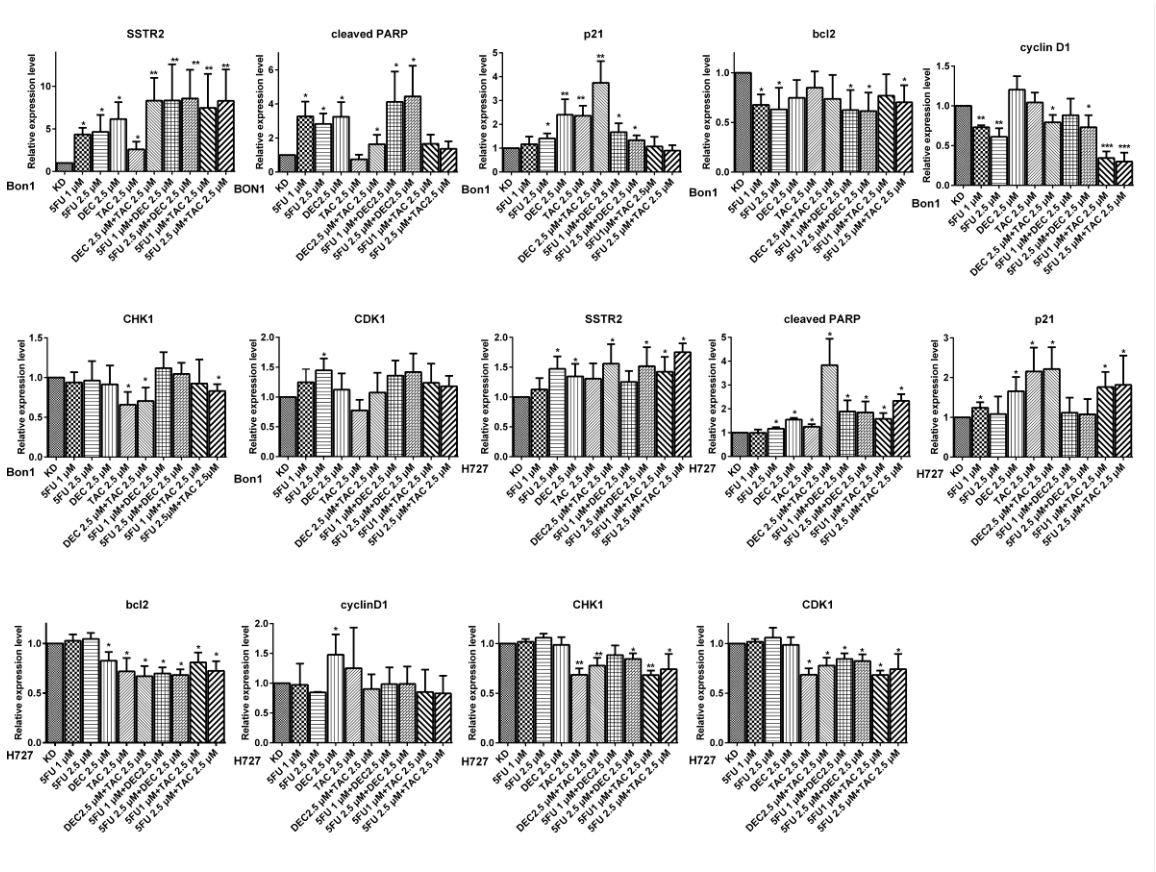

Fig 2-7. Representative quantitative westernblot of 5FU plus DEC or TAC on SSTR2, apoptosis and cell cycle related proteins in NET cells.

(relative changes in protein bands were measured by densitiometric analysis with the DMSO control，*p, **p and ***p stand for <0.05, <0.01, p<0.001 respectively).

2.3.7 Induction of SSTR2 expression after treatement with single drugs or their combination in NET cells

As shown in Fig.2.5, SSTR2 expression was promoted not only by DEC, TAC or 5FU alone, but further increased by 5FU+DEC, 5FU+TAC and DEC+TAC combinations in GOT1, Bon-1 and H727 cells, but not in QGP cells (Fig.2-6, Fig.2-7).

Thus, 5-FU plus DEC was the most effective in Bon-1 cells for upregulation of SSTR2 (8.56 fold increase), superior to that of 5-FU plus TAC (8.29 fold increase) or DEC plus TAC (8.31 fold increase), or either single drug (Fig.2-6 , Fig.2-7). In GOT1 cells, 5-FU plus TAC was also the most effective combination, whereas 5-FU plus DEC exerted a lesser effect than that of DEC plus TAC.

Moreover, in H727 cells, the highest efficacy was observed after combination of 5-FU plus TAC (1.75 fold increase), while 5-FU plus DEC (1.52 fold increase) and single drugs were slightly less effective (Fig. 2-6, Fig.2-7). Thus, these data suggest that SSTR2 upregulation might be cell-line dependent.

2.3.8 Effects of 5-FU combination with DEC or TAC on NET cell uptake of somatostatin receptor2

We next explored whether drug-induced SSTR2 expression might also cause an increased binding of 68Ga-DOTA-TOC to tumor cells.

Drug-induced SSTR2 upregulation was most profound in Bon1 cells followed by H727 cells. Therefore, the subsequent binding experiments, were performed in these two cell lines. Our data showed that not only 5-FU plus TAC but also 5-FU plus DEC time-dependently increase the uptake of 68Ga-DOTATOC without hitting a peak (Fig.2-8).

For the longest period of 4 h, a significant increase of uptake was observed in both 5-FU + DEC and 5-FU+TAC groups (13.0% ± 0.43% and 19.06% ± 1.1% vs. 2.96% ±0.21% of the control, $p<0.001$; respectively). The competitive non-labeled ^{68}Ga-DOTO-TATE blocking experiment revealed, that nonspecific uptakes of the 5-FU+DEC and 5-FU+TAC treated groups were only 0.48%±0.17%, 1.065%±0.23% respectively at 4 h.

Redundant qualitative results could also be observed in H727 cells, but in this cell line 5-FU plus DEC or 5-FU plus TAC revealed only an about twofold increase of 68Ga-DOTA-TOC intake (Fig. 2-8).

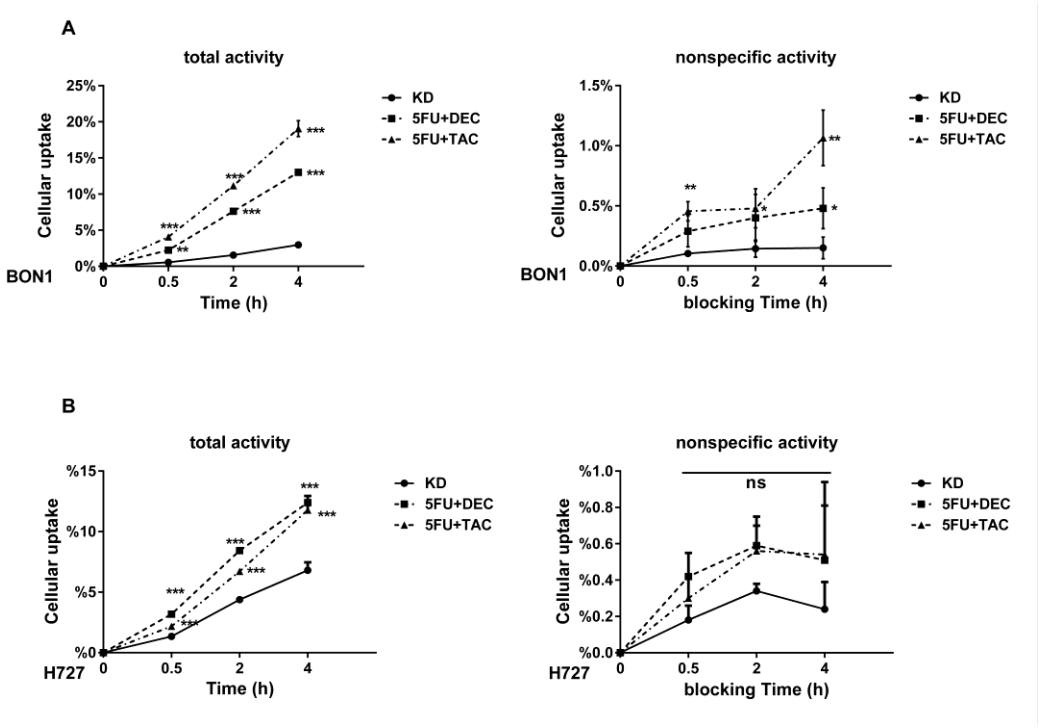

Fig. 2-8. 5-FU plus DEC or TAC promoted intake of SSTR2 in NET cells.

A(Bon1) and B(H727), the total internalized activity of 68Ga-DOTATOC. The intake is expressed as a percentage of added radioactivity (AR)/mg of protein. In each blocking experiment, 1,000 fold excess of the non-labeled ^{68}Ga-DOTO-TATE was added into cell culture. ***p<0.001, **p<0.01 and *p<0.05.

2.4 Discussion

PRRT with targeting radiopharmaceutical agents like 177Lutetium-DOTATOC or 177Lutetium- DOTATATE, has been mostly used to clinically manage NETs, but the objective response rates (ORRs) were only between 20% to 30% [114]. Thus, search for combination with other agents has been actively pursued including: i) radiosensitizing by 5-fluoropyrimidine based chemotherapies in clinical studies [26,86,104-105] or various molecular targeting therapy in preclinical studies [125-127], ii) epigenetic modification by DNA methyl transferase inhibitors (DNMTi), i.e. decitabine (DEC)] or histone deacetylase inhibitors (HDACi) i.e. tacedinaline (TAC)] to improve upregulation of SSTR[114-115]and radioligand binding [114-115] of the tumor cell in preclinical studies; iii) development of alpha targeted therapy rather than using the beta pharmaceuticals [128-130]; iv) using somatostatin antagonists rather than agonist [128,131].

In several clinical studies in patients with NETs further improvement of the therapeutic efficacy of PRRT has been reported by peptide receptor chemoradiation

therapy (PRCRT) [101] with fluoropyrimidine-based chemotherapeutic regimens using the combination of PRRT and chemotherapy with 5-fluorouracil [48], capecitabine [49], or capecitabin/ temozolomide [106,27].

Several preclinical studies from Taelman and Veenstra et al revealed that DNMTi and HDACi alone upregulate SSTR at the level of mRNA and protein expression, and enhance the SSTR2 mediated radioligand binding[114-115]. In addition, their combination showed addictive effects on increasing the SSTR2 expression and radioligand binding in animal models [114].

In the current preclinical in vitro study, we now systematically investigated the effects of the chemotherapeutic agent 5-FU alone and in combination with DEC or TAC on human NET cell lines with respect to reduction of cell viability; radiosensitizing; induction of apoptosis; promoting SSTR2 expression and increasing the radioligand binding of the tumor cell.

Our results demonstrate, that i) DEC or TAC dose-dependently reduce NET cell viability in vitro, while 5-FU in combination with DEC or TAC further synergistically

reduces NET cell viability; ii) 5-FU in combination with DEC or TAC is most efficient to reduce viability of gamma-irradiated NET cells in the colony formation assay, suggesting a significantly enhanced radiosensitizing effect in comparison to single drug treatment; iii) DEC and 5FU in particular resulted in great persistence of γH2AX-/53BP1 DNA damage after irradiation. iv) 5-FU in combination with DEC or TAC increases apoptosis subG1 events and induces expression of apoptosis-related proteins PARP or caspase-3 cleavage in NET cells; v) while 5-FU, DEC or TAC alone caused a modest increase in SSTR2 expression in NET cells, the combinations of DEC plus TAC as well as 5-FU plus DEC or 5-FU plus TAC induced a significant increase in SSTR2 expression in NET cells. vi) Binding assays with 68Ga-DOTA-TOC also demonstrated that 5-FU plus DEC or 5-FU plus TAC induced a significant increase of specific radioligand binding of NET cells. Thus, our current data suggest that the combination of 5-FU plus DEC or 5-FU plus TAC might be an efficient approach to enhance PRRT by combining radiosensitizing

effects and upregulation of somatostatin receptor (sstr) 2 expression and specific radioligand binding of the tumor cell.

5-FU is an analog of uracil and in cells, it will be incorporated into genomic DNA to inhibit the thymidylate synthase, leading to prevention of cells from synthesis of DNA, RNA, and protein [132,133]. 5-FU-induced tumor cell radiosensitization has been reported to be mediated by an increase in killing S phase cells [132]. However, other mechanisms may also mediate 5-FU-induced tumor cell radiosensitivity. In the current study, 5-FU could induce NET cell apoptosis and arrest NET cells at the G2 cell cycle and if 5-FU was combined with DEC or TAC, NET cell viability was synergistically reduced. Indeed, the combination of DNMT inhibitors and HDAC inhibitors has been already demonstrated to show synergistic antitumor effects [134]. This is because DNMT and HDAC inhibitors exhibited an overlapping role in alteration of gene expression and other cell functions. DNMTs or other methylated-DNA binding proteins could facilitate histone deacetylation by recruiting HDAC activity [135,136]. Molecularly, HDAC helps DNA methylation through

recruiting histone H3 lys9 methyltransferase [136]. The methylated genomic DNA will lose the transcriptional activity. In this regard, they could synergistically reduce cell activity in cell growth and gene expressions to enhance radiosensitivity [137,138].

In our current study, we expanded our observations showing that treatment of NET cells with 5-FU and DEC alone or 5-FU plus DEC induced NET cells to increase expression of cleaved PARP, cleaved caspase3, and p21waf1/cip1, but reduce anti-apoptotoc bcl2 protein expression, indicating induction of NET cell apoptosis. Our current finding is consistent with previous studies of HDAC inhibitors in NETs [110,139-140]. Furthermore, 5-FU is a cell cycle-dependent anticancer agent[141] and 5-FU plus DEC or 5-FU plus TAC also decreased expression of CyclinD1, CDK1/4/6, and CHK1 in this study. Thus, treatment of NET cells with 5-FU in combination with DEC or TAC could reach a synergistic effect on NET cells.

It has been demonstrated that the induction or upregulation of SSTR by DNMTi or HDACi was not only observed in human NET cell lines but also in human hepa-

tocellular carcinoma[142], pancreatic adenocarcinoma [143], small cell lung cancer [144] and prostate cancer LNCaP cells[145]. It has been established that HDACi or DNMTI regulated the activation and transcription of the upstream promoter of the human SSTR2 (hSSTR2). Meanwhile, ours is the first study to reveal that 5-fluorouacil or other fluoropyrimidine agents with or without DEC/TAC can also promote SSTR2 expression in NETs.

In summary and conclusion, these preclinical in vitro data suggest, that the combination of 5-FU plus DEC or 5-FU plus TAC might be an efficient approach to enhance PRRT by combining radiosensitizing effects and upregulation of somatostatin receptor (sstr)2 expression and specific radioligand binding of the tumor cell. Future clinical trials are essential to confirm the therapeutic efficacy of this novel strategy for peptide receptor chemoradiation therapy (PRCRT) combining the conventional chemotherapeutic drug 5-FU and an epigenetic modifier as the DNA methyl transferase inhibitor decitabine or the histone deacetylase inhibitor tacedinaline.

3. Conclusions and outlook

Chapter one:

Besides the suppression of canonical Wnt/β-catenin signaling pathway, the PORCN inhibitor WNT974 also inhibited the non-canonical wnt pathways, i.e.pEGFR, pIGFR and PI3K/AKT/mTOR pathways. Meanwhile, the antitumor activity of WNT974 was partially GSK3β-dependent and partially GSK3β-independent.

ß-catenin inhibitor PRI-724 also exerts potent antitumoral effects in NETs in vitro.

Molecular targeted therapy with wnt signaling inhibitors/beta-catenin inhibitors in NETs should be further explored in preclinical animal models and in early clinical studies. In the future next generation sequenzing (NGS) of tumors might provide predictive markers to select patients with predictive mutations (i.e. menin or APC) for personalized treatment.

Chapter Two:

Decitabine (DEC) or Tacedinaline (TAC) dose-dependently reduced NET cell viability in vitro; 5-FU combination with DEC or TAC synergistically reduced NET cell viability and colony formation; DEC and 5FU in particular resulted in great persistence of γH2AX-/53BP1 DNA damage foci after irradiation.5-FU or 5-FU plus DEC treatment induced a drastic rise of G2 phase with subsequent decline of S and G1 phase; 5-FU combination with DEC or TAC upregulated the expression of apoptosis-related markers cleaved caspase3 or cleaved PARP; Most importantly, 5-FU or 5-FU combination with DEC or TAC upregulated SSTR2 expression and induced the binding of ^{68}Ga-DOTA-TOC in NETs.

Future preclinical and clinical trials are urgent to confirm the therapeutic efficacy of this novel strategy for peptide receptor chemoradiation therapy (PRCRT) combining the conventional chemotherapeutic drug 5-FU and DNA methyltransferase inhibitors or histone deacetylase inhibitors in NETs.

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5. List of Abreviations

Abreviations	Full Name
PRRT	peptide receptor based radionuclide therapy
NET	Neuroendocrine tumor
pNETs	Pancreatic Neuroendocrine tumors
SI-NETs	Small Intestinal Neuroendocrine Tumors
FZD	Frizzled
LRP	Low-density-lipoprotein Receptor-related Protein
PORCN	Porcupine
CBP	CREB-binding Protein
PBS	Phosphate-Buffered Saline
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
ECL	Enhanced Chemiluminescence
ANOVA	One-Way Analysis Of Variance
SFRP1	Secreted Frizzled-Related Protein 1
MEN1	Multiple Endocrine Neoplasia type 1
NT	Neurotensin
CDKs	Cyclin-Dependent Kinases
AIF	Apoptosis Inducing Factor
DVL	Dishevelled
GSK3β	Glycogen Synthase Kinase 3 beta
TCF/LEF	TranscriptionFactor/LymphoidEnhancer-Binding Factor
DMSO	Dimethyl Sulfoxide
ZO-1	Zona Occludens 1
PI3K	Phosphoinositide 3-Kinase
mTOR	Rapamycin

RTKs	Receptor Tyrosine Kinases
MAPK	Mitogen-Activated Protein Kinase
APC	Adenomatous polyposis coli
EGFR	Epidermal growth factor receptor
IGFR	insulin-like growth factor receptor
PKB/AKT	Proteinkinase B
PKC	protein kinase C
S6K1	Ribosomal protein S6 kinase beta-1
4EBP1	Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
JNK	c-Jun N-terminal kinase
GSK3	Glycogen synthase kinase-3 (GSK3)
AXIN	Axis Inhibition Protein
5FU	5-Fluorouracil
ORR	Objective Response Rate
PRRT	Peptide Receptor Radionuclide Therapy
PRCRT	peptide receptor chemoradionuclide therapy
DNMT	DNA methyltransferase
DEC	Decitabine
TAC	Tacedinaline
HDAC	Histone deacetylase
SSTR2	Somatostatin Receptor type 2
SER	SensitizationEnhancement Ratios
NELMs	Liver Metastases of Neuroendocrine
OS	Overall Survival
DCR	Disease Control Rate
PFS	Progression-free survival

DMSO	Dimethyl Sulfoxide
PARP	Poly (ADP-ribose) polymerase
SF	Surviving Fractions
TBST	Tris buffer
ANOVA	One-Way Analysis Of Variance Test
CI	Combination Index
DSBs	DNA Double-Strand Breaks
γH2AX	gamma H2A histone family member X
53BP1	p53-Binding Protein 1
177Lu-DOA-TATE	177 Lu-DOTA0, Tyr3-octreotate
68Ga-DOTATOC	^{68} Ga-Edotreotide
γ-IR	gamma-irradiation
DNA	deoxyribonucleic acid
RNA	Ribonucleic Acid
CTNNB	Catenin, beta-1
Bcl-2	B-cell lymphoma 2
CHK1/2	Checkpoint Kinase 1/2
CKI	Cyclin-Dependent Kinase Inhibitor
CK1α	Casein Kinase 1α
DMEM	Dulbecco's Modified Eagle's Medium
RPMI	Roswell Park Memorial Institute medium
FBS	Fetal Bovine Serum
siRNA	small interfering RNA
ROR2	Receptor tyrosine kinase-like orphan receptor 2
PCP	Planar Cell Polarity
DKK1	Dickkopf-1
NT	Neurotensin

LQ	Llinear-quadratic
TSC1/2	Tuberous sclerosis tumor suppressor proteins hamartin1/2

6. List of Figures

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