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Schedule-dependent Interactions between Pemetrexed and Vinorelbine in Human Lung Cancer Cells

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Abstrakt -Zusammenfasung

Von den durch Krebs verursachten Todesfällen weltweit entfallen die meisten auf Lungenkrebs. Trotz Fortschritten und Weiterentwicklungen in der Chirurgie, der Chemotherapie und der Radiotherapie über die letzten Jahrzehnte hinweg blieb die Todesrate von Lungenkrebs weitgehend unverändert, was hauptsächlich auf die Metastasenbildung der Krankheit und Mehrfachresistenzen zurückzuführen ist. Aufgrund der allgemein schlechten Prognose werden neue Behandlungsstrategien für Lungenkrebspatienten dringend benötigt.

Das Ziel dieser Studie war es, Interaktionen zwischen Pemetrexed und Vinorelbin für menschliche Adenokarzinome mittels verschiedener Chemotherapieschemata zu untersuchen. Vinorelbin und Pemetrexed verursachten einen starken dosisabhängigen zytotoxischen Effekt sowohl bei HCC als auch bei HCC-res Zellen. Die IC_{50}-Werte von Vin gegenüber HCC bzw. HCC-res Zellen betrugen 10.34±1.12 nM bzw. 9.98±2.12 nM. Die IC_{50}-Werte von Pem gegenüber diesen Zellen betrugen 110.77±17.28 nM bzw. 118.89±18.77 nM.

Die Anwendung verschiedener Therapieschemata induzierte eine signifikante, zeitabhängige Hemmung des Zellwachstums bei unbehandelten HCC und cisplatin-resistenten HCC Zellen. Das Therapieschema von Cisplatin→Pemetrexed→Vinorelbin zeigte den stärksten inhibitorischen Effekt sowohl bei HCC als auch bei HCC-res Zellen.

Die Anwendung verschiedener Therapieschemata bei HCC und HCC-res Zellen erhöhte den Anteil von Apoptose betroffener Zellen, ausgenommen die alleinige Anwendung von Vinorelbin. Sowohl bei HCC als auch bei HCC-res Zellen stellte sich Cisplatin→Pemetrexed→Vinorelbin als effektivste Kombination zur Herbeiführung der Apoptose heraus.

Die Anwendung verschiedener Therapieschemata bei HCC und HCC-res Zellen erhöhte die Konzentration von Calcium. Nur die alleinige Anwendung von Vin führte nicht zu einer Erhöhung der Calcium Konzentration in HCC Zellen. Der höchste Anstieg von der Calcium Konzentration wurde in den mit Cisplatin→Pemetrexed→Vinorelbin behandelten HCC sowie auch HCC-res Zellen nachgewiesen.

Wir zeigten, dass die sequenzielle Anwendung von Cisplatin, Vinorelbin und Pemetrexed einen synergetischen Effekt bei der Hemmung des Zellwachstums, der Herbeiführung von Apoptose sowie der Erhöhung von der Calcium Konzentration gleichwohl in HCC und HCC-res Zellen ausübt. Die Überfrachtung mit Calcium könnte zur Apoptose führen, was mit dem Inhibitionseffekt auf das Zellwachstum von Chemotherapeutika bei Lungenkrebszellen in Zusammenhang steht. Es liefert möglicherweise einen Beitrag zur Entwicklung chemotherapeutischer Ablaufpläne für Patienten und zur Überwindung von Cisplatin-Resistenz bei Lungenkrebs.

Abstract - Summary

Lung cancer is the leading cause of cancer deaths worldwide. Despite advances and progresses in surgery, chemotherapy, and radiotherapy over the last decades, the death rate from lung cancer has remained largely unchanged, which is mainly due to metastatic disease and multi drug resistance. Because of the overall poor prognosis, new treatment strategies for lung cancer patients are urgently needed. The aim of this study was to investigate the interactions between pemetrexed and vinorelbine for human adenocarcinoma via various chemotherapy schedules.

Vinorelbine and pemetrexed caused a strong dose-dependent cytotoxic effect in both HCC and cisplatin resistant HCC (HCC-res) cells. The IC_{50} values of vinorelbine against HCC and HCC-res cells were 10.34±1.12 nM and 9.98±2.12 nM, respectively. The IC_{50} values of Pemetrexed against these cells were 110.77±17.28 nM and 118.89±18.77 nM respectively. The application of different therapy schedules induced a significant time dependent cell growth inhibition on HCC naïve and cisplatin resistant cells. The therapy scheme of cisplatin→pemetrexed→vinorelbine showed the strongest inhibitory effect on both HCC and HCC-res cells.

The application of different therapy schedules on HCC and HCC-res cells increased the percentage of cells undergoing apoptosis, except the application of vinorelbine alone. In both HCC and HCC-res cells, cisplatin→pemetrexed→vinorelbine was found the most effective to induce apoptosis.

The application of different therapy schedules on HCC and HCC-res cells increased cytoplasma calcium concentration. Only the application of vinorelbine alone failed to increase calcium concentration in HCC cells. The most elevated calcium concentration was found in the cells treated with cisplatin→pemetrexed→vinorelbine in both HCC and HCC-res cells

As a conclusion, the sequential application of cisplatin, vinorelbine and pemetrexed has a synergistic effect in cell growth inhibition, apoptosis induction, and calcium concentration elevation in HCC and HCC-res cells. The calcium overload could lead to apoptosis, which was related to the cell growth inhibitory effect of chemotherapeutics in lung cancer cells. It might cast a light to develop chemotherapy schedules for patients, and to overcome cisplatin resistance in lung cancer.

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1 INTRODUCTION

1.1 Lung cancer and lung cancer therapy

Lung cancer is the leading cause of cancer deaths worldwide. The two major forms of lung cancer are non–small cell lung cancer (NSCLC, about 85% of all lung cancers) and small-cell lung cancer (SCLC, about 15%) [1]. Lung cancer causes more deaths than the next three most common cancers combined (colon, breast and prostate), accounting for approximately 28% of all cancer deaths [2]. The lung cancer five-year survival rate (16.3%) is lower than many other leading cancer sites, such as the colon (65.2%), breast (90.0%) and prostate (99.9%), which accounts 52.6% for cases detected when the disease is still localized however only 15% of lung cancer cases are diagnosed at an early stage [3]. For distant tumors the five-year survival rate is only 3.5% and over half of people with lung cancer die within one year of being diagnosed [4].

Overall, women have an improved outcome compared with men across all ages and irrespective of histological subtype. A decrease in the age-adjusted lung cancer-related mortality in males has been observed since the 1990s. In females, the rate has reached a plateau after a long period of increase between the mid-1960s and 1995 [5]. Whether women are more vulnerable to tobacco carcinogens remains controversial. Women smokers are more likely than men to develop lung adenocarcinomas, and women who have never smoked are more likely to develop lung cancer than men who have never smoked. This difference has been attributed to estrogen signaling [6].

The treatment and prevention of lung cancer are major unmet needs that can be improved by further investigation of more effective therapeutic plan with a better understanding of the molecular origins and evolution of the disease.

Despite advances in early detection and standard treatment, NSCLC is often diagnosed at an advanced stage with a result in a poor prognosis [7]. Histologically NSCLC can be divided into three major subtypes: squamous-cell carcinoma, adenocarcinoma, and large-cell lung cancer. Smoking is related to all types of lung cancer but is most strongly linked with SCLC and squamous-cell carcinoma; adenocarcinoma is the most common type in patients with no smoking history [8]. Small cell lung carcinoma (SCLC) is an aggressive form of lung cancer

that is strongly associated with cigarette smoking and has a tendency for early dissemination [9].

Environmental factors, such as tobacco smoke, and genetic susceptibility interact to influence carcinogenesis, as well as other factors including genetic, hormonal, and viral factors. Occupational or environmental exposure to second-hand smoke, radon, asbestosis, and radiation represent other risk factors [10]. Tissue injury initially occurs in the form of genetic and epigenetic changes (e.g. mutations, loss of heterozygosity, and promoter methylation) and global transcriptome changes (e.g., inflammation and apoptosis pathways). These changes can persist long term and eventually lead to aberrant pathway activation and cellular function, such as dysregulated proliferation and apoptosis, to produce premalignant changes, including dysplasia and clonal patches. The changes can result in angiogenesis, invasion and early-stage cancer, and advanced cancer and metastasis [11].

Progresses have been made for defining cancer risk, prognosis, and optimal therapy aimed at personalized prevention and treatment of lung cancer with the development of molecular techniques and biomarkers [12]. Lung cancer is initiated by activation of oncogenes or inactivation of tumor suppressor genes. Mutations in the K-ras proto-oncogene are present in 10–30% of lung adenocarcinomas [13]. The epidermal growth factor receptor (EGFR) regulates cell proliferation, apoptosis, angiogenesis, and tumor invasion. Mutations and amplification of EGFR are common in NSCLC and provide the basis for treatment with EGFR-inhibitors [10]. A variant in the nicotinic acetylcholine receptor was found to be associated with nicotine dependence. Other candidates include 5p15.33, 15q25.1, and 6p21.33. Spitz et al. identified an association between an intronic SNP in the ACVR1B gene and cancer susceptibility in never smokers exposed to second-hand smoke by using a pathway-based approach and focusing on single-nucleotide polymorphisms (SNP) in inflammatory-pathway genes [14]. The p53 tumor suppressor gene, located on chromosome 17p, is affected in 60-75% of cases. Other genes that are often mutated or amplified are c-MET, NKX2-1, LKB1, PIK3CA, and BRAF [15]. There are multiple genetic and epigenetic changes in lung cancer, but in some types a specific driving genetic change is found like an activating EGFR mutation or and EML4-ALK overexpression.

Risk models as well as other risk factors could eventually allow the identification of high-risk populations that might be good candidates for screening programs. Several randomized controlled studies have investigated the relationship between tobacco smoking as a major risk

factor and the impact of chest x-ray screening [16]. None of them has shown a reduction in lung cancer mortality, although it allows detection of early stage and asymptomatic lung cancers. Oken *et. al*. has recently reported that annual screening with chest X-ray was not found to be able to reduce lung cancer mortality as compared with usual care[17]. CT-scan, as significantly more sensitive tool than chest X-ray for identifying small and asymptomatic lung cancers, was evaluated in The National Lung Screening Trial (NLST). A low dose CT-scan in high risk individuals (current smokers with cigarette smoking history of more than 30 pack-years, and former smokers who quit within the previous 15 years) was carried out. Participants who received low-dose CT scans had a 20% lower risk of dying from lung cancer than participants who received standard chest X-rays [18]. Several other studies are ongoing to validate the clinical benefit of this observation, but many questions remain to be solved in terms of frequency, duration, and cost-effectiveness of CT-scan before that evidence translates into policy and practice. Rapid progress and availability of whole genome sequencing, genome-wide analysis approaches may also uncover more targetable lesions in lung cancer, such as the K-Ras proto-oncogene [19].

Despite advances and progresses in surgery, chemotherapy, and radiotherapy over the last decades, the death rate from lung cancer has remained largely unchanged, which is mainly due to metastatic disease. Because of the overall poor prognosis, new treatment strategies for lung cancer patients are urgently needed.

1.1.1 Non-small cell lung cancer

NSCLC includes squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. NSCLC are frequently resistant to drug therapy and obtaining a complete response is rare. Therefore, the drug resistance in patients receiving chemotherapy alone or when combined with radiotherapy represents a major problem in cancer treatment of patients with NSCLC [7].

While there are several therapeutic options for the management of NSCLC, the disease is often not diagnosed until the advanced stage at which point therapy is not curative but to extend and improve quality of life (QOL) [20]. In 70% of the cases, patients are diagnosed with stage IV amenable only to systemic therapy. Combination chemotherapy is the current standard of care for patients with advanced NSCLC. NICE guideline recommended two-drug combination chemotherapy with a single third generation drug, such as pemetrexed, docetaxel,

gemcitabine, paclitaxel or vinorelbine, plus a platinum (carboplatin or cisplatin) for patients with preserved function status; single-agent therapy with a third generation drug should be given to the patients unable to tolerant a platinum-based combination [21]. Cisplatin is associated with slightly improved response rates, with no overall improvement of survival, and a different toxicity profile compared with carboplatin. In two meta analyses cisplatin was superior in overall survival in patients with non-squamous histology [22].As a major progress in recent years, Bevacizumab, a VEGF monoclonal antibody, has been approved in combination with carboplatin and paclitaxel in patients with non-squamous cell carcinoma and good performance status, negative for brain metastasis, and without hemoptysis or therapeutic anticoagulation [23]. In the FLEX study, cetuximab, an EGFR monoclonal antibody, has shown to improve survival in combination with cisplatin and vinorelbine in patients positive for EGFR expression identified via immunohistochemistry [10]. High EGFR expression has been recently reported as a predictive biomarker of survival benefit from the addition of cetuximab in K-Ras mutation in colon cancer [24].

There is growing evidence indicating that accessory cells, also referred as stromal cells, play an essential role in disease progression in various cancers, including lung cancer. Regulated migration and homing of stem cells to tissue niches is a critical step during embryonic development or tissue repair, but also in cancer (stem-) cell dissemination [25]. A complex network of growth factors, cytokines, and chemokines is employed by the tumor cells in order to organize the microenvironment. Key players in tumor-microenvironment cross talk are mesenchymal stromal cells (MSC), monocyte/macrophage lineage cells, and T lymphocytes, along with extracellular matrix and blood vessels. Navab et al. demonstrated that carcinoma-associated fibroblasts (CAFs) display a greater ability than normal fibroblasts to enhance the tumorigenicity in NSCLC [26]. 46 genes were identified overexpressed in CAFs via gene profiling, encoding for proteins that are involved in TGF-β signaling, focal adhesion, and the MAPK signaling pathway. Cells of monocytes/macrophage lineage are another highly important cell type in the tumor microenvironment. Tumor-associated macrophages (TAM) support lung cancer progression by inducing cancer cell motility and metastasis, and angiogenesis [27]. Among many factors released by TAM, CXCL12 is a prominent mediator accounting for TAM accumulation. An animal model recently highlighted the importance of peroxisome proliferator-activated receptor-γ (PPARγ) in TAM for promoting NSCLC progression and metastasis. Increased numbers of regulatory T cells (Treg) in the tumor microenvironment are thought to suppress immune recognition and immune response to the

neoplastic cells, thereby promoting tumor progression. NSCLC infiltration by Tregs was associated with earlier relapses, especially in patients with node-negative NSCLC [28].

1.1.2 Small cell lung cancer

SCLC is an aggressive form of lung cancer that is strongly associated with cigarette smoking and has a tendency for early dissemination. Although most of the patients respond to chemotherapy, motility rate is still 95% of the patients [9]. SCLC accounts 14% of newly diagnosed lung cancer cases. The vast majority, more than 95%, of SCLC patients have smoking history [29].

The specific sequence of genetic alteration of SCLC tumorigenesis remains unclear, however the genetic and molecular changes have been found to be related to SCLC: 1) Autocrine growth loops. Autocrine of gastrin-releasing peptide, neuromedin B, tyrosine kinase receptor c-kit have been found to be related to SCLC tumorigenesis. 2) Proto-oncogenes. The overexpression of MYC proteins in SCLC lead to more rapid proliferation and loss of terminal differentiation. 3) Tumor-suppressor genes. The loss of alleles from the short arm of chromosome 3 is the most common deletion of tumor DNA in SCLC, including genes as FHIT, RASSFIA, TP53, RB1 and PTEN [8].

Patients with SCLC typically present with disseminated diseases. Symptoms include local ones such as cough, dyspnoea, chest pain, haemoptysis, and hoarseness, and distant ones such as weight loss, weakness, anorexia, paraneoplastic symptoms, and fever. SCLC tends to be centrally located with hilar masses and hilar and mediastinal adenopathy. Diagnosis is typically made by histological analysis of a bronchoscopic biopsy or percutaneous or transbronchial aspiration samples [29].

Combination chemotherapy remains the major treatment for SCLC. The administration of four to six cycles etoposide and cisplatin plus chest radiotherapy for patients with good performance status and limited stage disease produces a complete-response rate of 80% or even higher, median survival excess of 17 months, and 5-year tumor-free of 12-25% [30]. Combination chemotherapy remains the focus of treatment for patients with extensive-stage diseases, which leads to a complete-response rate of about 20% and median survival of about 7 months. Changes in combination of chemotherapy medications and schedule of

chemotherapy cycle have been investigated. Some trials have shown slight survival benefits favoring the regimens with three or four drugs but at the cost of greater toxicity. The use of weekly chemotherapy resulted in greater toxicity with no survival advantage. And neither shortened interval between cycles nor maintenance therapy was found to provide consistent benefit for the patients treated [9].

Most patients with small-cell lung cancer relapse within a year of starting treatment. The likelihood of response to subsequent therapy can be predicted on the basis of the response to previous therapy and the duration of drug-free interval. Patients who did not respond to previous therapy or who relapsed within 3 months of completing therapy are judged refractory, whereas those who had responded to previous treatment and relapsed 3 months or longer after completing such treatment are deemed sensitive.

1.2 Multi-drug resistance

Multidrug resistance (MDR) is defined as a protection of the cells against numerous drugs, with different chemical structures and by different intracellular functional mechanisms, like Anthracyclines (doxorubicin, daunorubicin, et al.), Epipodophyllotoxins (etoposide, teniposide, et al.), Vinca alkaloids (vincristine, vinblastine, et al.), Taxanes (paclitaxel, docetaxel, et al.), Kinase inhibitors (imatinib, flavopyridol, et al.) and other preparation classes. MDR turns into an extraordinary limitation to cancer chemotherapy. There are different mechanisms involved in MDR, including:

Abnormal expression of membrane protein drug pumps. The ABC transporters ABCB1, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5 and ABCG2 have been discovered to be implicated in MDR. So far ABCB1 (also known as Pgp) has been found to transport the largest number of drugs. The same drug can be a substrate of different ABC transporters while ABC transporters have distinct substrate specificities [31, 32].

Abnormality of intracellular enzyme systems. DNA topoisomerase (Top) is a ribozyme for DNA replication and transcription. Down-regulation of Top expression and change in the type of expression are important reasons for SCLC resistance to Top inhibitors.

Abnormality of cell apoptosis. Apoptosis escape in tumor cells starts from the activation of anti-apoptotic pathways via extracellular signals; insensitization of the endogenous cell death machinery via resisting apoptotic protein “addiction”; and loss of apoptosis-promoting tumor suppressor genes via accumulated mutations. Integrinsplay an important role in inducing cell apoptosis and blocking metastasis. In cell adhesion mediated drug resistance, extracelluar matrix (ECM) protein can resist apoptotic signals induced by cytotoxic drugs. In more than 80% SCLC cases, a loss of p53 activity has been found.

Enhancement of cell repair systems. Studies in recent years show that DNA mismatch repair (MMR) plays a very important role in the progression of SCLC chemoresistance. Down-regulation of MMR gene MLH1 and MSH2 may be associated with the occurrence and MDR of lung cancer. There may be a link to silencing of MMR genes induced by acetylation and phosphorylation of histones and hypermethylation of promoters.

The resistance of tumour cells to chemotherapy agents can be caused by a number of cellular adaptations, including reduced uptake, inactivation by glutathione and other anti-oxidants, and increased levels of DNA repair or DNA tolerance. Chemotherapy agents, including cisplatin, pemetrexed, and vinorelbine, are transported in tumour cells via ABC transporters, the over activation and/or over expression can result in chemotherapy resistance. Treatment with these agents is characterized by resistance, both acquired and intrinsic. For instance, the overexpression of cMOAT (MRP2/ABCC2) is associated with decreased formation of platinum-DNA adducts and decreased G2-arrest in melanoma cells resistant to cisplatin [33]. Pemetrexed gains cell entry via several of ABC transporters, and other mechanisms. The resistance to pemetrexed can occur in a number of circumstances, such as catalytic activity or expression of FPGS alteration, membrane transport alternation, reducing the availability of free antifolate substrate to react with FPGS, increased cellular levels of natural folates, and increased activity of γ-glutamyl hydrolase [20]. Vinorelbine is transported via efflux pump ABCC10&MRP7 and RLIP76, a non-ABC transporter, to conferring drug accumulation defect and resistance in lung cancer cells. Bessho et. al. found the gene expression of ABCB1/MRP1 and ABCC10/MRP7 in vinorelbine resistant NSCLC cell lines [34].

1.3 New therapeutic strategy

There is a rich drug development pipeline of novel chemotherapeutic agents for lung cancer such as amrubicin, SABA, picoplatin, belotecan and vinflunine. Amrubicin is a synthetic anthracycline that blocks DNA repair by inhibiting Top I [35-37]. Clinical trials have shown that the response rate of combined use of amrubicin and picoplatin is as high as 88%, and the median survival is 13.6 months [7]. Picoplatin is a platinum analogue that overcomes platinum drug resistance, with lower ototoxicity and nephrotoxicity than those of other platinum drugs [38].

Some molecular targeted drugs have entered clinical trials, including matrix metalloproteinase (MMP) inhibitors, thalidomide, biological vaccines, and small molecular weight inhibitors directed at receptor protein tyrosine kinases such as EGFR, c-Kit and VEGFR [39]. Unfortunately, the therapeutic effects are unsatisfactory. MMPs regulate extracellular matrix modelling, maintaining cell growth and morphology. Up-regulation of MMP is considered a marker for poor prognosis of SCLC [36]. However, some large-scale randomized phase III clinical trials suggest that use of MMP inhibitor marimastat and tanomastat as maintenance therapy after failing induction therapy did not seem to prolong the survival of NSCLC patients [40]. C-Kit protein has been found highly expressed in lung cancer, to stimulate cell growth signalling pathways in an autocrine or paracrine manner [41]. Imatinib can inhibit activities of c-Kit receptor tyrosine kinase, bcr/ abl fusion protein and platelet-derived growth factor receptor (PDGFR) family tyrosine kinase [42]. KIT gene can encode transmembrane tyrosine kinase growth factor receptor of the PDGFR family. However, phase II clinical trials have shown that imatinib did not prolong progression-free survival (PFS) of sensitivity and drug resistance-induced relapse of SCLC patients with high expression of c-Kit [43]. And no additional therapeutic effect was observed after treatment on the basis of the platinum plus irinotecan schedule. Thalidomide is a multi-target angiogenesis inhibitor, that inhibits vascular endothelial growth factor (VEGF), fibroblast growth factor β (FGFβ) and tumor necrosis factor α (TNFα), and modify intracellular matrix [44]. Phase III clinical trials using thalidomide on the basis of carboplatin+etoposide and using thalidomide for maintenance therapy showed satisfactory tolerance and response [45]. Pravastatin also exhibited its inhibitory effect on SCLC growth and sensitizing effect on chemotherapeutic agents. Targeted mitochondrial apoptosis pathway is an SCLC drug resistance-reversing strategy. It was found in a phase I clinical trial that the Bcl-2 targeted inhibitor Oblimersen relieved 86% of untreated ES-SCLC cases [46]. It was found in a pre-clinical study that drug resistance to

ABT- 263 in a SCLC cell line and a transplanted tumor model was accompanied with the increased concentration of apoptosis-promoting protein Bax, Bim and NOXA [6]. A combined use of spindle toxic drugs and apoptosis-targeted inhibitors may produce a synergistic pro-apoptotic effect through double inhibition of anti-apoptotic protein Mcl-1 and Bcl-xl [47]. It has been demonstrated in SCLC cell line in vitro and NSCLC transplanted tumor models that combined use of Bcl-xl targeted inhibitor Navitoclax (ABT-263) and spindle toxic drugs could induce apoptosis of tumors cells in mitotic arrest phase [48].

1.4 Pemetrexed

Pemetrexed (Alimata®) is a multi-targeted antifolate cytotoxic agent approved in Europe and US as first line therapy in combination with cisplatin, and as monotherapy after prior chemotherapy, or as maintenance monotherapy, for patients with local advanced or metastatic non-squamous or predominant non-squamous NSCLC [49]. It is applied in combination with cisplatin in patients with malignant pleural mesothelioma, bladder, breast, cervical, colorectal, gastric, head and neck, and pancreatic cancers.

Pemetrexed is a pyrrolo [2, 3-d] pyrimidine-based antifolate and has antineoplastic effects by disturbing folate-dependent metabolic processes essential for cellular replication. Pemetrexed inhibits thymidylate synthase (TS) and at a lower efficacy for other secondary enzyme targets, including glycinamide ribonucleotide formyltransferase, aminoimidazolecarboxamide ribonucleotide formyltransferase (AICARFT), and rapamycin pathway, which is responsible for the balance of cellular energy metabolism, protein and lipid synthesis and growth) [27]. Thymidylate synthase plays an important role in DNA biosynthesis by catalysing the methylation of fluorodeoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). High TS expression levels correlate with key mechanisms of antimetabolite drug resistance. TS expression levels predicted survival in untreated NSCLC patients and postoperative recurrence of pulmonary adenocarcinomas. An association of TS expression with response to S-1 plus carboplatin in advanced NSCLC was also reported recently [50].

Pemetrexed gains cellular entry via the reduced folate transporter, the α-folate receptor, and proton-coupled folate transporter. Intracellular pemetrexed is polyglutamated to its active form by folylpolyglutamate synthetase (FPGS), which results in accumulation of pemetrexed

derivatives with greater affinity for its enzyme targets mentioned above than the parent drug [20]. Induction of apoptosis, modulation of EGFR and Akt phosphorylation, and alternations in the expression of critical tumorigenesis genes involved in the activity of pemetrexed are mechanistic contributors to the synergistic interaction.

Pemetrexed has a steady-state volume of distribution of approximately 16 L. It is approximately 81% bound to plasma proteins in vitro. It undergoes minimal hepatic metabolism. The total systemic clearance is around 92 ml/min and the plasma elimination half-life is 3.5 h in patients with normal renal function. The drug is predominantly eliminated unchanged in the urine, with between 70% and 90% of a dose recovered unchanged within 24 h of administration [51].

The efficacy of pemetrexed in patients with NSCLC has been demonstrated in several clinical trials, in combination with cisplatin as first-line therapy, monotherapy as second-line therapy, and as maintenance monotherapy. Pemetrexed plus cisplatin was non-inferior to gemcitabine plus cisplatin with median overall survival was 10.3 months with both treatments [52]. But pemetrexed did appear to have survival advantages in patients with non-squamous diseases. In patients whose disease had not progressed with four cycles of first-line platinum-based therapy, median progression-free survival and overall survival times in patients who received pemetrexed plus best supportive care were significantly longer than placebo control group. It has been suggested in different studies that the treatment paradigm may provide QOL advantage for the patients. Most trials that investigated the duration of first-line platinum-based therapies have received equivalent survival between the shorter and longer therapy durations. Immediate maintenance therapy after four cycles of platinum-based therapy is a promising approach [50].

Pemetrexed is generally well tolerated and the tolerability profile is favourable as combination therapy with cisplatin and also as monotherapy in patients with advanced NSCLC. Adverse events have been reported as neutropenia, skin rash, nausea or vomiting, mucositis and diarrhoea.

1.5 Vinorelbine

Vinorelbine (Navelbine®) is a semisynthetic vinca alkaloid derivative that demonstrates strong antitumor activity in various malignancies. It is indicated as a single agent or in combination with cisplatin for the first-line treatment of ambulatory patients with unresectable, advanced NSCLC [39]. It is also applied in patients with breast cancer, SCLC, ovarian carcinoma, cervical carcinoma, B-cell malignancies, Hodgkin's disease, and multiple myeloma.

It exerts a cytotoxic effect upon rapidly proliferating tumors through prevention of mitotic spindle formation by inhibiting the polymerization of tubulin into microtubules. The vinca alkaloids are structurally similar compounds comprised of 2 multiringed units, vindoline and catharanthine, with the catharanthine unit being the site of structural modification for vinorelbine [53]. The antitumor activity of vinorelbine is thought to be due primarily to inhibition of mitosis at metaphase through its interaction with tubulin. It may also interfere with amino acid, cyclic AMP, and glutathione metabolism, calmodulin-dependent calcium transport ATPase activity, cellular respiration, and nucleic acid and lipid biosynthesis [54].

The pharmacokinetic properties of intravenously administered vinorelbine are: after a dose of 30 mg m–2 i.v. a high initial peak of 5 μmol rapidly decays to about 1 nmol at 2 h. Distribution in blood is rapid, with binding of 78% of the drug to platelets and a further 13.5% to plasma proteins with only 1.7% left as free drug in the first 2 h after administration. Subsequently, binding to plasma proteins is in the order of 70–80% [55]. The drug diffuses freely into tissues showing a large volume of distribution and an elimination half-life of 40 h. 30 minutes after administration, high levels of vinorelbine are found in both normal lung and tumour tissue and diffusion out of tumour tissue appears to be slow. The metabolism of vinorelbine is principally hepatic and only 11% of the drug is excreted via the renal route, the majority being eliminated through faecal excretion [56].

Vinorelbine is widely utilized in combination chemotherapy for NSCLC. In different clinical studies, the cisplatin vinorelbine combination showed a superior response-rate of 30% compared to 19% for cisplatin-vindesine and 14% for vinorelbine alone; 1-year survival rates were 35%, 27% and 30% respectively. Combined chemotherapy and radiotherapy is a common approach in the treatment of NSCLC. Cisplatin-vinorelbine was used as the induction regimen in patients with stage III-B disease prior to radical radiotherapy with an

objective response of 48%, leading the authors to conclude that this is an effective regimen pre-radiotherapy [57].

1.6 Calcium signalling

1.6.1 Calcium channels and pumps

The calcium ion is a ubiquitous cellular signal, which regulates various cellular processes by activating or inhibiting cellular signalling pathways and calcium-regulated proteins, such as gene transcription, muscle contraction, synaptic transmission, cell proliferation and apoptosis [58]. Cells have to shape calcium signals in the dimensions of time, space, and amplitude in order to carry out the calcium controlled cell biology activities.

Calcium channels, pumps and exchangers control the complex and tight regulation of calcium homeostasis, which differ in their cellular distribution and their mechanism of transport. A strict equilibrium between the 'on' and 'off' mechanisms in the cells keeps calcium under regulation within cellular compartments to achieve the sensitive control of cell signalling pathways that can precisely respond to many stimuli. Resting cytoplasm free Ca^{2+} ($[Ca^{2+}]_c$) is maintained at a low level (~100nM), with a much higher extracellular Ca^{2+} concentration of ~1.2 mM [59]. The calcium 'on' mechanisms include the plasma membrane (PM) channels, which regulate the calcium supply from extracellular space, the endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR) channels [60]. An equally set of 'off' to remove calcium from the cytoplasm includes calcium ATPases on the PM and ER/SR, and additionally to exchangers that utilize gradients of ions to provide the energy to transport calcium out of the cell, such as Na^+/Ca^{2+} exchange. Mitochondria also play an important role in the regulation of cytoplasm calcium. These calcium stores have a low affinity but high-capacity rapid calcium uniporter that can significantly reduce cytoplasmic calcium transients and diminish cellular responses.

Within the cell there is a calcium concentration gradient between the cytoplasm and the Ca^{2+} stores. The calcium stores are enriched with calcium binding proteins, such as calsequestrin and calreticulin. Ca^{2+} can be released from the stores by the generation of inositol 1, 4, 5-trisphosphate (IP3) [61]. IP3 is highly mobile in the cytoplasm and diffuses into the cell

interior where it encounters specific IP3 receptors (IP3Rs) on ER/SR. The binding of IP3 changes the conformation of IP3Rs so that an integral channel is opened, thus allowing the Ca^{2+} in the ER/SR to enter the cytoplasm. IP3Rs are composed of four subunits (~1200 kDa), encoded by three different genes [46]. The opening of IP3Rs is enhanced by the modest increase of Ca^{2+} concentration (0.5-1 μM) whereas higher Ca^{2+} concentration (> 1 μM) inhibits the opening. Ryanodine receptors (RyRs) are structurally and functionally analogous to IP3Rs, with an approximately twice the conductance and molecular mass of IP3R [62]. RyRs are generally actived by the increase of Ca^{2+} of 1-10 μM and inhibited by higher Ca^{2+} concentration of > 10 μM. They are largely present in excitable cell types [63]. The opening of these channels has been shown to be modulated by numerous factors, including phosphorylation, adenine nucleotides, thiol reactive compounds, pH level and the Ca^{2+} load of ER/SR [60]. Other than the Ca^{2+} channels on ER/SR, there are also Ca^{2+} ATPase pumps which actively transport Ca^{2+} against a concentration gradient, such as the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases (SERCAs) pumping Ca^{2+} into the ER.

There are mainly three types of calcium entry channels: (1) receptor-operated calcium channels (ROCCs), which comprise a range of structurally and functionally diverse channels. They are particularly prevalent on secretory cells and at nerve terminals. ROCCs are activated by the binding of an agonist to the extracellular domain of the channel [64]. (2) voltage-operated calcium channels (VOCCs), which are largely employed by excitable cell types such as muscle and neuronal cells, where they are activated by depolarisation of the PM. (3) store-operated calcium channels (SOCCs), which are activated in response to depletion of the intracellular calcium stores, either by physiological calcium -mobilising messengers or pharmacological agents. SOCCs are the most ubiquitous PM calcium channels. The mechanisms of how the SOCCs sense the status of the intracellular pool are unknown.

1.6.2 Calcium and cancer

Since calcium signalling can affect pathways regulating proliferation and apoptosis, alterations in calcium channels and pumps could have a causal and promoting role in cancer. The location, degree and temporal aspects of changes in cytoplasm calcium concentration regulate the pathways relating to tumorigenesis.

Alternations in calcium channels and pumps are detected in many cancers. SOCCs as IP3R2 and IP3R3 were found over expressed in non-small cell lung cancer and gastric cancer respectively [24, 65]. Down-regulation of SERCA 2 was discovered in oral cancer [66], colon cancer [67], thyroid cancer [68] and lung cancer [67] but up-regulation in colorectal cancer [69]. SERCA 3 was found down regulated in colon cancer [70]. Changes in the expression or activity of PMCA pumps and transient receptor potential channels, such as TRPM8, TRPM1, TRPV1, TRP6, and TRPC6, were also found in several types of cancers [61].

Calcium is implicated in cellular motility including neurite outgrowth and contraction [58, 71]. Calcium, is also a key regulator of signalling pathway important in angiogenesis, since some angiogenic stimuli, such as vascular endothelial growth factor, can increase cytoplasm calcium concentration by mobilizing calcium release from the internal calcium stores [72]. Calcium can influence genomic stability and cell survival, for example calcium is a modulator of poly (ADP-ribose) polymerase-1 (PARP1) activity, which alters cellular metabolism and DNA repair [73]. Calcium is a key regulator of the cell cycle, and hence proliferation, through various different pathways including Ras signalling [74]. Calcium signalling is implicated in the cancer cell differentiation process either through the extracellular calcium-sensing receptor and/or through changes in intracellular calcium [75]. Calcium can modulate cell-cycle regulators directly, for example by activating the transcription of the genes crucial in the G0-G1 transition [76] and for the phosphorylation of retinoblastoma protein in late G1 phase [77]. Calcium can also indirectly regulate the subcellular localization of the key tumorigenic proteins. Minaguchi et al. found out that the nuclear localization of PTEN is regulated by Calcium through a tyrosil phosphorylation-independent conformational modification in major vault protein [76]. The accumulation of excessive calcium has often been found to link to apoptosis and necrosis by the activation of ER/SR and mitochondrial membrane permeabilization [78]. A reduction of ER calcium content is associated with resistance to apoptosis [46].

1.6.3 Target calcium in cancer

Calcium channels and pumps with altered expression and/or activity in cancer might represent potential biomarkers of disease. Changes in the ER calcium pump SERCA3 protein expression is either reduced or lost in colon carcinomas compared with normal tissue,

consistent with a loss of differentiation in cancer cells [79]. The increased apoptotic resistance of the malignant neuroendocrine differentiated prostate cells is due to a general alteration in calcium homeostasis in which the reduction in SERCA2b has an important role. The reduced ER calcium content partially because of the decrease of SERCA2b expression is a probable mechanism for apoptotic resistance [80]. These calcium channels and pumps can be used as prognostic indicators or can guide treatment by the means of techniques such as microarrays in cancers.

Calcium channels and pumps with altered expression offer the potential not only as biomarker for cancer diagnosis but also anticancer therapeutic targets. So modulating the activity of calcium channels and pumps that are aberrantly expressed in cancer cells and cancer stem cells efficiently might sufficiently interrupt calcium homeostasis to target cancer cells with restricted tissue distribution.

The significance of increased expression of calcium channels and pumps in cancer is directly related to the calcium regulated tumorigenic pathways. Many of the calcium channels and pumps with altered expression in cancer have a highly restricted tissue distribution, unlike many of the ubiquitous potential anticancer drug targets such as cell cycle regulator. Therapies based on a target with a limited tissue distribution are less associated with generalized toxicity, which is a factor limiting clinical use for agents that have widespread expression. For instance, PMCA2 is up-regulated in human breast cancer cell lines, whereas its expression is restricted normally to the central nervous system [81].

The availability of pharmacological calcium channels and pumps inhibitors or activators makes it as an outstanding feature of calcium channels or pumps as cancer targets. For instance heparin, dantrolene and CPA are inhibitors for IP3R, RyR and SERCA, while adenophostin A and suramin are activators for IP3R and RyR (SERCA activator has not been discovered) [82-84].

2 METHODS AND MATERIALS

2.1 Biological material

Tumor cell lines

HCC-78: DSMZ no. ACC 563, human non-small cell lung carcinoma. According to the histological classification of the original tumor, HCC is defined as adenocarcinoma (Virmani et. al., 1998) and characterized as cytokeratin +, cytokeratin-7 +, cytokeratin-8 +, cytokeratin-17 +, cytokeratin-18 +, cytokeratin-19 +, desmin -, endothel -, EpCAM +, GFAP -, neurofilament -, vimentin +.

HCC-78 cisplatin resistant cell line: generated from HCC-78 cell line via cisplatin treatment selection, and maintained with low dose cisplatin in culture. It has been developed by continuously exposing cells to gradually increasing doses of cisplatin and use of the limiting dilution technique [85].

2.2 Lab material and equipment

2.2.1 Laboratory consumables

- Multi-well dishes: 96-well and 6-well, Cat. 353072, 353935 and 351146, Falcon, BD Biosciences Labware, NJ, US.
- Cell culture dishes: 35 × 10 mm and 100 × 20 mm, Cat. 353001 and 353003, Falcon, BD Biosciences Labware, NJ, US.
- Centrifuge tubes: 15 ml and 50 ml, Cat. 62 554 502 and 227 261, Sarstedt AG & Co., Nümbrecht, Germany.
- Cryotubes: Cryo Vials, Cat. 121 277, Greiner Bio-One GmbH, Frickenhausen, Germany.

- Filter system: 500 ml, 0.22 µm filter, Cat. 430758, Corning Incorporated, NY, US
- Slides: 25 × 1.0 × 75 mm, superfrost, Cat. J1800AMNZ, Menzel-Gläser, Mezel GmbH & Co KG, Braunschweig, Germany.
- Cover slides: 24 × 32 mm and 18 × 18 mm, Menzel-Gläser, Mezel GmbH & Co KG, Braunschweig, Germany.
- Counting chamber: 0.1 × 0.0025 mm2, Cat. 63510-10, Neubauer, Brand, Germany.
- Pipettes: 10 ml, Cat. 47110, Sterilin Ltd. Caerphilly, UK; 25 ml, Cat. 4251, Corning Incorporated, US.
- Tips: 10 µl, 200 µl, 1000 µl, Cat. 70 1115, 70 760 002 and 70 762, Sarstedt AG & Co., Nümbrecht, Germany.
- Culture flasks: 25 cm^2, 2 µm vent cap, Cat. 430639, Corning Incorporated, NY, US; 75 cm^2 and 175 cm^2, 2 µm vent cap, Cat. 658175 and 660175, Greiner Bio-One GmbH, Frickenhausen, Germany.

2.2.2 Lab equipment

- Thermomixer: 5436, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany.
- Magnetic stirrer: IkaMag RH, Janke & Kunkel IKA-Labortechnik, Staufen, Germany.
- Centrifuge: Hettich EBA 12R and Universal 16A, Minnesota, US.
- Eppendorf pipettes: 0.5-10 µl, 10-100 µl, 20-200 µl, 100-1000 µl, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany.
- Electronic pipette controller: Gilson, Middleton, US.
- Water bath: Techne TE-10D, Tempunit Gesellschaft für Laborgeräte mbH, Wertheim, Germany.

2.3 Cell culture

2.3.1 Cell culture medium and supplement

- RPMI 1640: Cat. F1415, Biochrom AG, Berlin, Germany.
- Trypsin-EDTA: 0.05%/0.02% in D-PBS, Cat. L11-004, PAA Laboratories GmbH, Pasching, Austria.
- Fungizone: amphothericin B, 250 µg/ml, Cat. P11-001, PAA Laboratories GmbH, Pasching, Austria.
- Penstrep: penicillin (10,000 IU/ml) and streptomycin (10,000 IU/ml), Cat. P11-010, PAA Laboratories GmbH, Pasching, Austria.
- L-glutamine: 200 mM, Cat. M11-004, PAA Laboratories GmbH, Pasching, Austria.
- FBS: REF. 16170-078, Gibco, Darmstadt, Germany.
- PBS: Phosphate Buffered Saline, without Ca^{2+} and Mg^{2+}, Cat. H15-002, PAA Laboratories GmbH, Pasching, Austria.
- Ethanol: pure, Pharmacy, Klinikum Grosshadern, Munich, Germany.
- DMSO: dimethyl sulfoxide, 5 ×10 ml. Cat. D2650, Sigma-Aldrich, St. Louis, US.
- Trypan blue: 0.4%, Cat. T8154, Sigma-Aldrich, St. Louis, US.
- Cisplatin: 1 mg, Pharmacy, Klinikum Grosshadern, Munich, Germany.
- Pemetrexed: 1 mg, Pharmacy, Klinikum Grosshadern, Munich, Germany.
- Vinorelbine: 1 mg, Pharmacy, Klinikum Grosshadern, Munich, Germany.

2.3.2 Cell culture equipment

- Laminar airflow: Heraeus, Munich, Germany.
- Incubator: Heraeus, Munich, Germany.

2.3.3 Cell culture medium recipe

- HCC medium

  Final Conc.

90%	RPMI 1640
10%	FBS heat inactivated
200 mM	L-glutamine
10,000 IU/ml	Penstrep
2.5 μg/ml	Fungizone

2.3.4 Monolayer culture of lung cancer cell line HCC

HCC cells were cultured in 175 cm^2 culture flasks with 25 ml medium and cultured in the 37 °C cell incubator with 5% CO_2. Every 2-3 days the culture medium was changed and they were split with Trypsin-EDTA solution and incubated in the cell incubator for 5 min when the cells were 60-70% confluent. The cells were frozen in liquid nitrogen with 10% DMSO culture medium for future use.

2.3.5 Survival curve

HCC cells (1×10^5) were seeded in 25 cm^2 cell culture flask and cultured for 24 h, and afterward treated with pemerexed and vinorelbine. For cisplatin treatment, the chemotherapy drug was added 3 h before pemetrexed or vinorelbine. The cell viability was evaluated after the cells were exposed to the treatment for 24 h, 48 h, 72 h and 96 h by trypan blue exclusion cell counting. This approach was chosen because after application of cisplatin to human, a relevant plasma concentration of unbound cisplatin (active form) persists for only 3 h [60].

2.4 Apoptosis measurement via flow cytometry

2.4.1 Material and equipment

- FACSAria II flow cytometer: BD, New Jersey, USA.
- Alexa Fluor: 350 annexin V conjugate, Cat. A23202, Invitrogen, Darmstadt, Germany.
- Propidium iodide: 1 mg/ml, Cat. P4170, Sigma-Aldrich, St. Louis, US.

2.4.2 FACS analys

Single-cell suspensions of HCC cells, 1×10^6/ml in 2% FBS RPMI 1640, were treated with cisplatin, pemetrexed and vinorelbine according to the schedule for 48 h. The cells were harvested after the incubation period and wash in cold PBS. Re-centrifuge the washed cells, discard the supernatants, and then resuspend the cells in annexin-binding buffer. Determine the cell density and dilute in annexin-binding buffer to ca. 1×10^6 cells/mL, preparing a sufficient volume to have 100 µl per assay. Add 5 µl of the annexin V conjugate to each 100 µl of cell suspension. Incubate the cells at room temperature for 15 minutes. Propidium iodide was added with a final concentration of 5 µg/10^6 cells to discriminate viable and non-viable cells before measurements. Cells were analyzed with FACSAria II flow cytometer as soon as possible. The population separated into at least two groups: live cells with only a low level of fluorescence and apoptotic cells with substantially higher fluorescence intensity.

2.5 Calcium staining

2.5.1 Calcium staining material

- Fura-2 calcium imaging calibration kit: 11 Ca^{2+} standard buffers premixed with 50µM fura-2, Cat. F-6774, Molecular Probes, Invitrogen, Eugene, US.

- Fura-2 AM: 50 μg × 20 special packaging, Cat. F14185, Molecular Probes, Invitrogen, Eugene, US.

2.5.2 Composition of fluorescent microscope for calcium imaging

- Microscope: Axiovert 200M, SIP 79800, Carl Zeiss AG, Jena, Germany.
- HBO lamp: 103W/2, short Arc mercury lamp, Osram GmbH, Augsburg, Germany.
- Objective Fluar: 20 × 0.75, transmission wavelength from 340 nm, Zeiss AG, Oberkochen, Germany.
- Filters: excitation wavelength 340 nm and 380 nm, emission wavelength 510 nm both, Cat. 340 AF 15 and 380 AF 15, Laser components GmbH, Olching, Germany.
- CCD digital camera: AxioCam MRm, Carl Zeiss Vision, Munich, Germany.

2.5.3 Fura-2 calibration curve

A ratio metric measurement method was utilized to determine the dissociation constant (Kd) of Fura-2 AM at the staining condition, in order to reduce artifacts from the microscope application and to generate the absolute cytoplasm calcium concentration [86].

With the Fura-2 calcium imaging calibration kit, images of different calcium concentration standard solution with Fura-2 AM were acquired, with excitation at 340 nm and 380 nm respectively, while the emission was measured at 510 nm, according the protocol offered with the kit. With the online Kd calculator (www.probes.com), the Kd of Fura-2 AM was yielded as 269 nM [87]. (Figure 1)

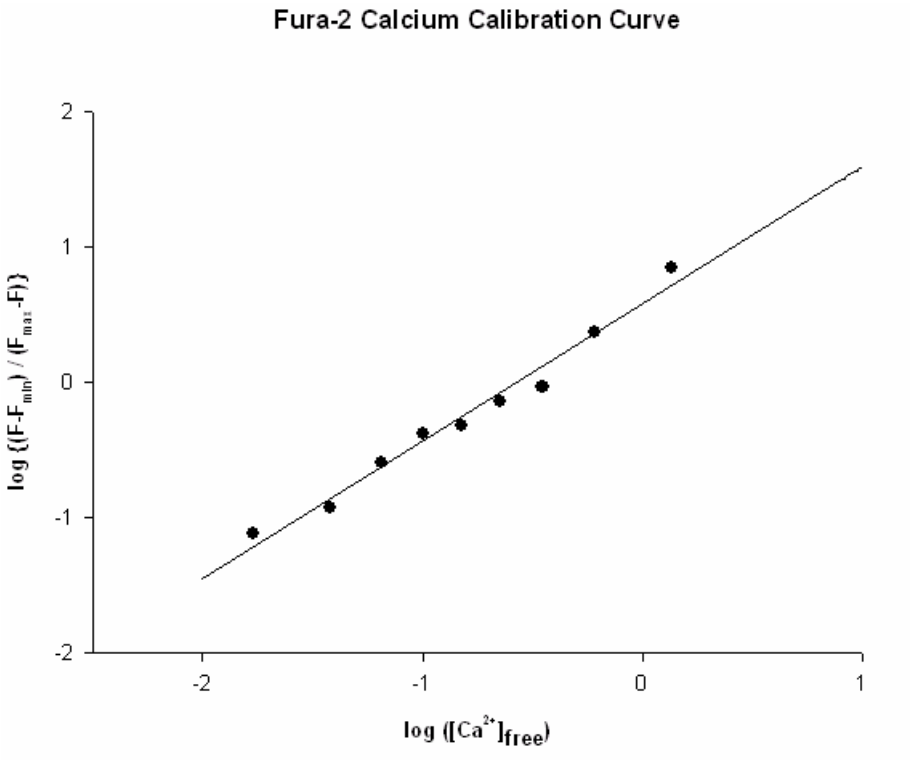


Figure 1. Fura-2 calcium calibration curve: the Kd of Fura-2 AM was yielded as 269 nM.

A ratio (R) of emission intensities was calculated as the emission intensity at 510 nm from 340 nm excitation divided by the emission intensity at 510 nm from 380 nm excitation ($R = F^{340}/F^{380}$). According the equation below, the cytoplasm free calcium concentration can be obtained from the fluorescence emission intensity ratio:

$$[Ca^{2+}]_{free} = K_d \times \left[\frac{R-R_{min}}{R_{max}-R}\right] \times \left[\frac{F^{380}{}_{max}}{F^{380}{}_{min}}\right]$$

2.5.4 Cytoplasm calcium concentration measurement

HCC cells were seeded in cell culture dishes with the same density. After the cells grew adherent, Fura-2 AM was applied with a final concentration of 10 μM in 37°C cell incubator

for 90 min. After loading, the cells were incubated another 30 min in PBS (with Ca^{2+} and Mg^{2+}) to allow complete dye deesterification and they were examined with the fluorescent microscope.

Images of the same field were taken at both exciting wavelength 340 nm and 380 nm with the image-processing program Axio Vision 4.1 (Carl Zeiss). For each image, ROIs were defined in cytoplasm of every single cell, and the average fluorescence of ROIs was measured with the image-processing program Scion Image 4.0 (Scion). Cytoplasm calcium concentration was calculated as described above.

2.6 Statistics

One-way or two-way ANOVA (combined with pairwise multiple comparisons) were performed using Sigma Stat software (Jandel Scientific, Chicago, USA). A *p*-value of less than 0.05 was considered statistically significant.

3 RESULTS

3.1 Inhibitory effect of vinorelbine and pemetrexed on cell growth

Adeno lung carcinoma cells HCC and cisplatin resistant cells HCC-res were treated with vinorelbine and pemetrexed in a dose range of 0.1 nM up to 10 µM and 1 nM up to 100 µM respectively. Both HCC and HCC-res cells showed sensitivity to vinorelbine and pemetrexed. Vinorelbine and pemetrexed caused a strong dose-dependent cytotoxic effect in both HCC and HCC-res cells.

In HCC cells, survival was inhibited down to 7.57% after 24 h exposure to vinorelbine at the highest concentration, while HCC-res cells to 9.98%. In HCC cells, survival was inhibited down to 30.30% after 24 h exposure to pemetrexed at the highest concentration, while HCC-res cells to 35.42%.

The IC_{50} values of vinorelbine against HCC and HCC-res cells were 10.34±1.12 nM and 9.98±2.12 nM, respectively. The IC_{50} values of pemetrexed against these cells were 110.77±17.28 nM and 118.89±18.77 nM respectively. (Figure 2)

Figure 2A

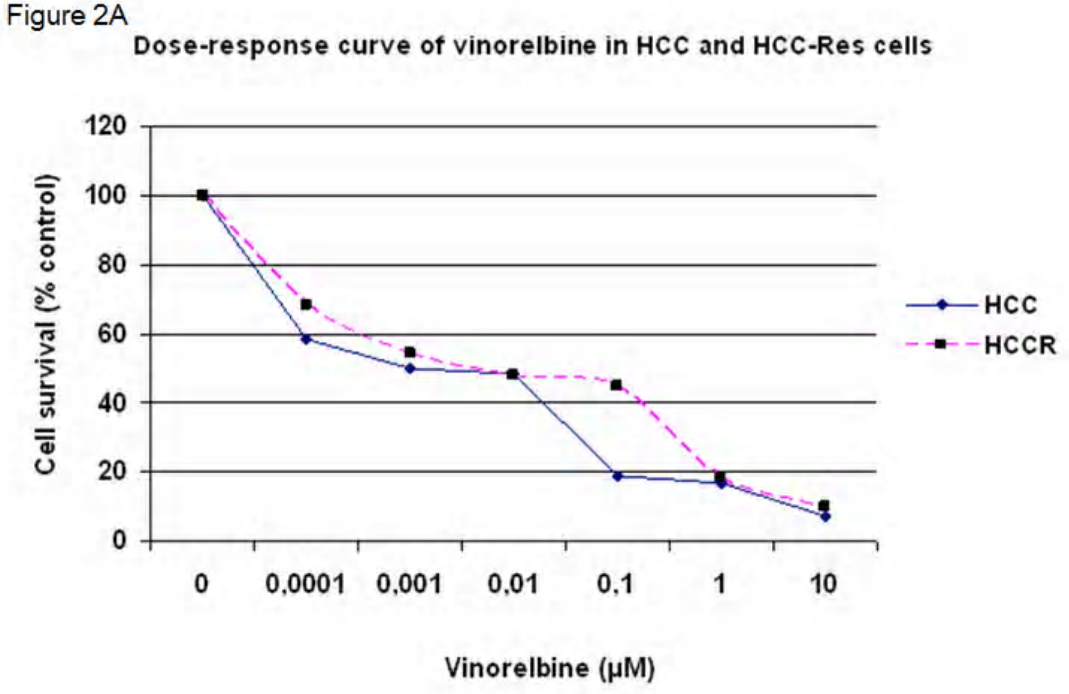


Figure 2B

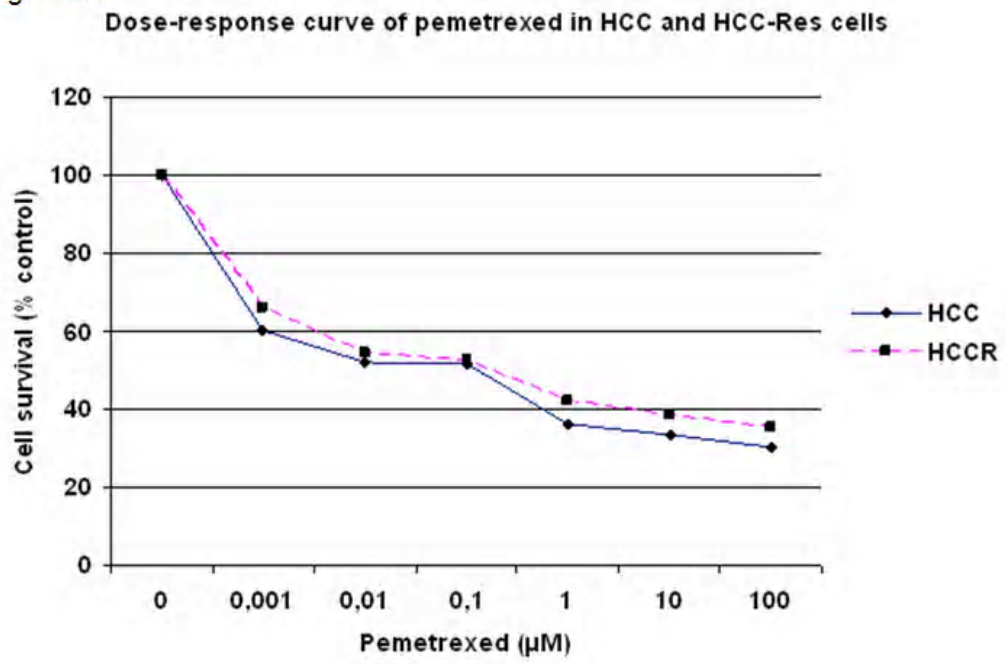


Figure 2. Inhibitory effect of vinorelbine and pemetrexed on cell growth: HCC and HCC-res cells were treated with different concentrations of vinorelbine and pemetrexed for 24 h. Error bars are not shown for clarity. Both HCC and HCC-res cells showed sensitivity to vinorelbine and pemetrexed. Vinorelbine and pemetrexed caused a strong dose-dependent cytotoxic effect in both HCC and HCC-res cells. (n=3)

3.2 Effect of vinorelbine, pemetrexed and different chemotherapy schedule on cell growth

HCC cells were treated with different schedule of combination of 1 µM cisplatin, 12 nM vinorelbine, and 130 nM pemetrexed respectively and the survival fraction was calculated every 24 h for 4 days after treatments. The schedules included single drug treatments (Cis, Vin, Pem), double drug combined treatments (Cis→Vin, Cis→Pem, Vin+Pem, Vin→Pem, and Pem→Vin), and triple drug combined treatments (Cis→Vin+Pem, Cis→Vin→Pem, Cis→Pem→Vin). The survival rate in all treatment groups were calculated with non-treatment control group.

In all treated groups, a significant time dependent cell growth inhibition could be shown. In HCC cells, the cell number decreased after 24 h treatment, the lowest survival fraction was

observed in the group treated with Cis→Pem→Vin at 96 h after the treatment as 1.17%±0.35%. (n = 3) (Figure 3A)

Figure 3A

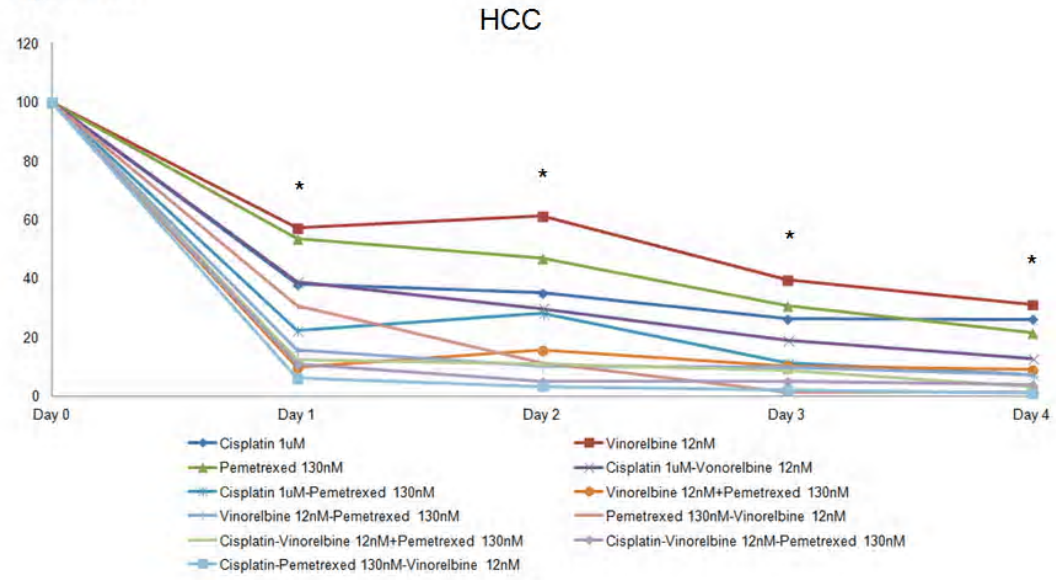


Figure 3A. Effect of vinorelbine, pemetrexed and different chemotherapy schedule on cell growth (all groups) in HCC cells: The schedules included single drug treatments (Cis, Vin, Pem), double drug combined treatments (Cis→Vin, Cis→Pem, Vin+Pem, Vin→Pem, and Pem→Vin), and triple drug combined treatments (Cis →Vin+Pem, Cis →Vin→Pem, Cis→Pem→Vin) for 4 days. In all treated groups, a significant time dependent cell growth inhibition could be shown. Error bars are not shown for clarity. (n=3, * = $P<0.05$ versus other experimental groups)

An additional inhibition effect was shown when the survival fraction in the combination of vinorelbine, pemetrexed and cisplatin treated groups were compared with that of cisplatin administrated alone.

The combination of Cis→Vin and Cis→Pem resulted in 12.67±2.51% and 7.28±2.02% survival fraction at 96 h after treatment respectively, comparing with exposure alone (31.20±6.92% of Vin, 21.57±3.61% of Pem and 26.23±6.22% of Cis).

Meanwhile when HCC cells treated with a triple drug schedule, the schedule of Cis→Pem→Vin exhibited the strongest inhibitory effect at 96 h after treatment with a survival fraction of 1.17%±0.35%. In the groups of Cis→Vin+Pem, and Cis→Vin→Pem, the survival fraction at 96 h after treatment were 3.38±1.23% and 3.99±1.07% respectively. (n = 3) (Figure 3B)

Figure 3B

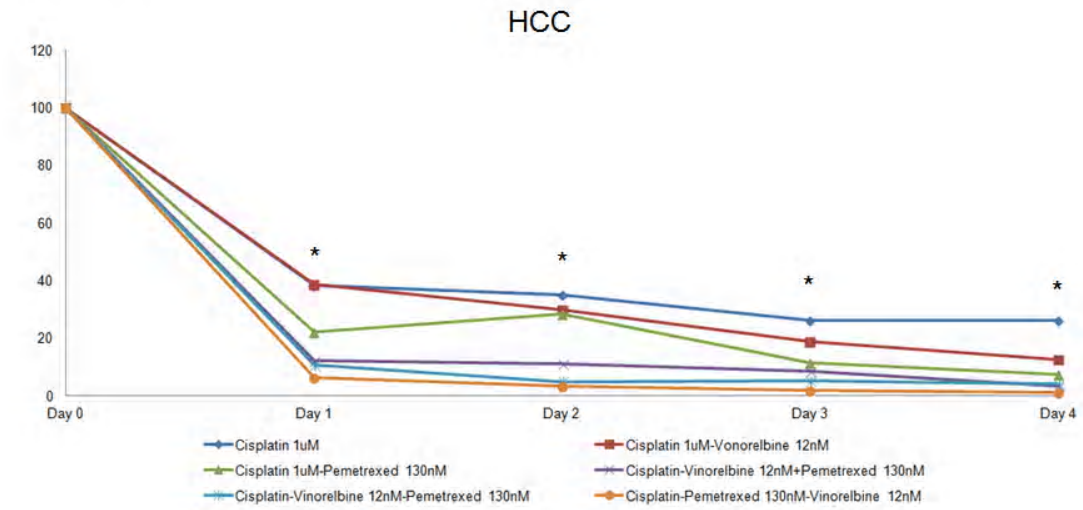


Figure 3B. Effect of vinorelbine, pemetrexed and different chemotherapy schedule on cell growth (groups include cisplatin in the schedule) in HCC cells: HCC cells treated with Cis, Cis→Vin, Cis→Pem, Cis→Vin+Pem, Cis→Vin→Pem, Cis→Pem→Vin for 4 days. An additional inhibition effect was shown when the survival fraction in the combination of vinorelbine, pemetrexed and cisplatin treated groups were compared with that of cisplatin administrated alone. Error bars are not shown for clarity. (n=3, * = $P<0.05$ versus other experimental groups)

Cisplatin resistant HCC cells HCC-res were treated with different schedule of combination of cisplatin, vinorelbine, and pemetrexed respectively and the survival fraction was calculated every 24 h for 4 days after treatments. The schedules were applied the same as those of HCC cells. The survival rate in all treatment groups were calculated with non-treatment control group. Cis did not show cell growth inhibitory effect in HCC-res cells.

In all other treated groups, a significant time dependent cell growth inhibition could be shown, and the cell number decreased after 24 h treatment with the lowest survival fraction observed in the group treated with Cis→Pem→Vin at 96 h after the treatment as 2.30%±0.37%. (n = 3) (Figure 4A)

Figure 4A

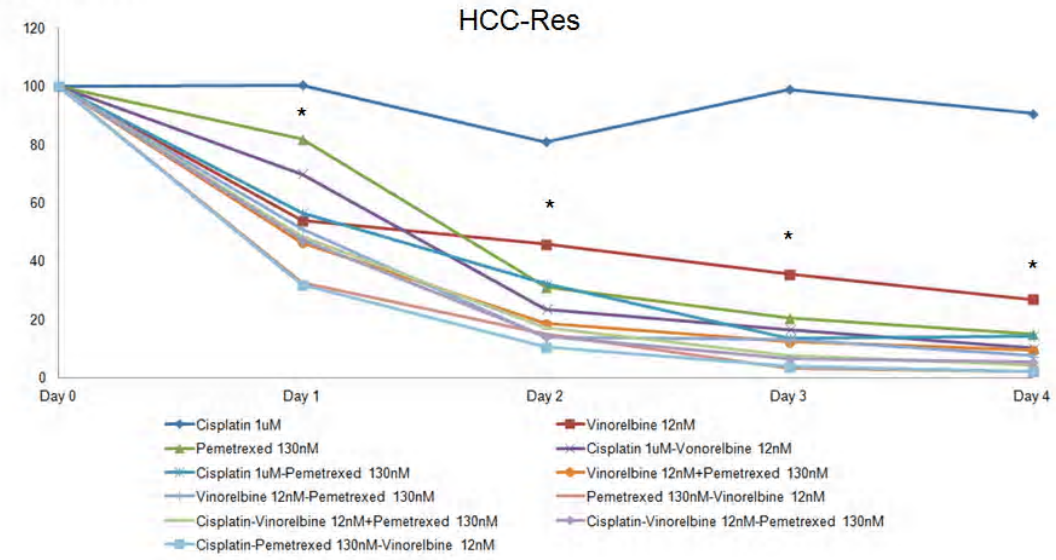


Figure 4A. Effect of vinorelbine, pemetrexed and different chemotherapy schedule on cell growth (all groups) in HCC-res cells: HCC-res cells treated with included single drug treatments (Cis, Vin, Pem), double drug combined treatments (Cis→Vin, Cis→Pem, Vin+Pem, Vin→Pem, and Pem→Vin), and triple drug combined treatments (Cis→Vin+Pem, Cis→Vin→Pem, Cis→Pem→Vin) for 4 days. In the treated groups except Cis treated group, a significant time dependent cell growth inhibition could be shown. Error bars are not shown for clarity. (n=3, * = $P<0.05$ versus other experimental groups)

A significant stronger inhibition effect was shown when the survival fraction in the combination of vinorelbine, pemetrexed and cisplatin treated groups were compared with that of cisplatin administrated alone or the control group.

The combination of Cis→Vin, Cis→Pem resulted in 10.27±2.82% and 14.43±2.62% survival fraction at 96 h after treatment respectively, comparing with exposure alone (26.97±7.91% of Vin, 15.11±2.68% of Pem and 90.54±3.29% of Cis).

Meanwhile when HCC cells treated with a triple drug schedule, the schedule of Cis→Pem→Vin exhibited the strongest inhibitory effect at 96 h after treatment with a survival fraction of 2.30%±0.65%. In the groups of Cis→Vin+Pem, and Cis→Vin→Pem, the survival fractions at 96 h after treatment were 4.28±0.33% and 5.48±0.87% respectively. (n = 3) (Figure 4B)

Figure 4B

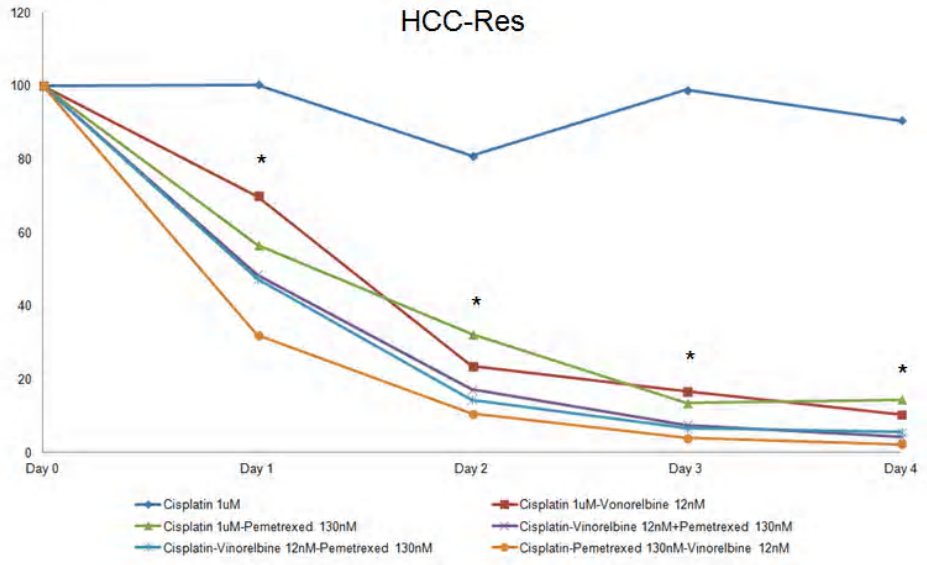


Figure 4B. Effect of vinorelbine, pemetrexed and different chemotherapy schedule on cell growth (groups include cisplatin in the schedule) in HCC-res cells: HCC-res cells treated with Cis, Cis→Vin, Cis→Pem, Cis→Vin+Pem, Cis→Vin→Pem, Cis→Pem→Vin for 4 days. An inhibition effect was shown when the survival fraction in the combination of Vin, Pem and Cis treated groups were compared with that of Cis administrated alone. Error bars are not shown for clarity. (n=3, * = $P<0.05$ versus other experimental groups)

3.3 Effect of vinorelbine, pemetrexed and different chemotherapy schedule on apoptosis

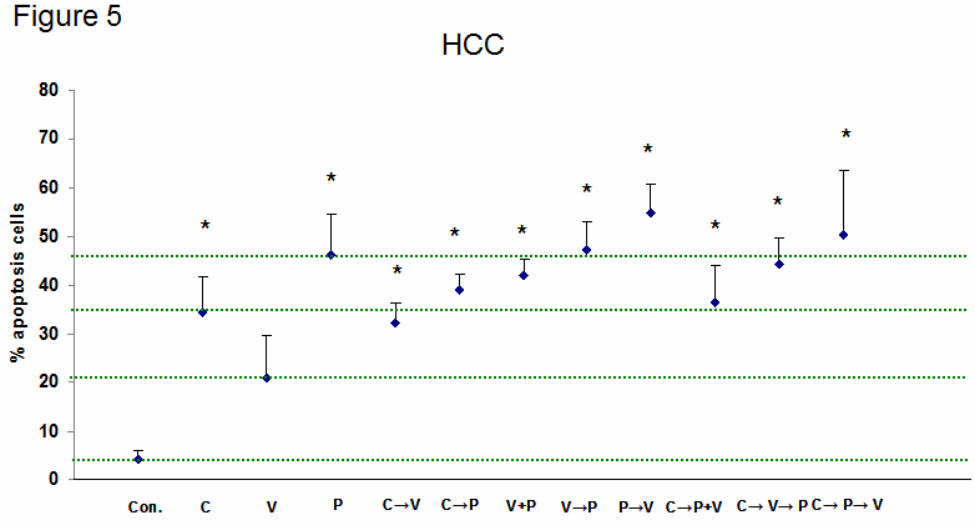
In order to investigate the effect of 12 nM vinorelbine, 130 nM pemetrexed and combined schemes on programmed cell death in HCC cells, different therapy schedule has been applied on HCC and HCC-res cells with or without cisplatin treatment. Apoptosis was measured after 48 h.

In HCC cells, the percentages of apoptotic cells in all treated groups except vinorelbine treated group were significantly higher than that of the control group. Single drug exposure of cisplatin, vinorelbine, and pemetrexed resulted in 34.48±7.04%, 20.94±8.75% and 46.00%±8.62% cell apoptosis respectively.

Sequential double drug application of Cis→Vin, or Cis→Pem resulted in 32.48±3.65% and 39.01±3.20% cell apoptosis respectively. Simultaneous exposure of Vin+Pem resulted in 41.85±3.44% cell apoptosis while sequential application of Vin→Pem or Pem→Vin as 47.45±5.52% and 54.91±5.97%.

Most interestingly, in the schemes of triple drug treatments, the highest apoptosis percentage was found in Cis→Pem→Vin as 50.28±13.18%, which was comparable to Pem→Vin. (n = 3) (Figure 5)

Figure 5



Fiure 5. Effect of vinorelbine, pemetrexed and different chemotherapy schedule on apoptosis in HCC cells: apoptosis was analysed after the cells were treated with different schedule of combination of cisplatin, vinorelbine, and pemetrexed respectively for 24 h. The percentages of apoptotic cells in all treated groups except vinorelbine group were significantly higher than the control group. (n=3, * = P<0.05 versus other experimental groups)

In HCC-res cells, apoptosis was analysed after the cells were treated with different schedule of combination of 1 μM cisplatin, 12 nM vinorelbine, and 130 nM pemetrexed respectively for 24 h. Cisplatin treatment showed no effect on apoptosis in HCC-res cells. The percentages of apoptotic cells in all other treated groups except vinorelbine were significantly higher than that of the control group. Single drug exposure of vinorelbine and pemetrexed resulted in 23.37±3.07%, and 42.94%±6.85% cell apoptosis respectively.

Sequential double drug application of Cis→Vin or Cis→Pem resulted in 28.39±5.38% and 44.00±5.29% cell apoptosis respectively. Simultaneous exposure of Vin+Pem resulted in 43.65±6.07% cell apoptosis while sequential application of Vin→Pem or Pem→Vin as 40.31±2.79% and 37.17±10.92%.

In the schemes of triple drug treatments, the highest apoptosis percentage was found in Cis→Pem→Vin as 51.88±9.74%. (n = 3) (Figure 6)

Figure 6

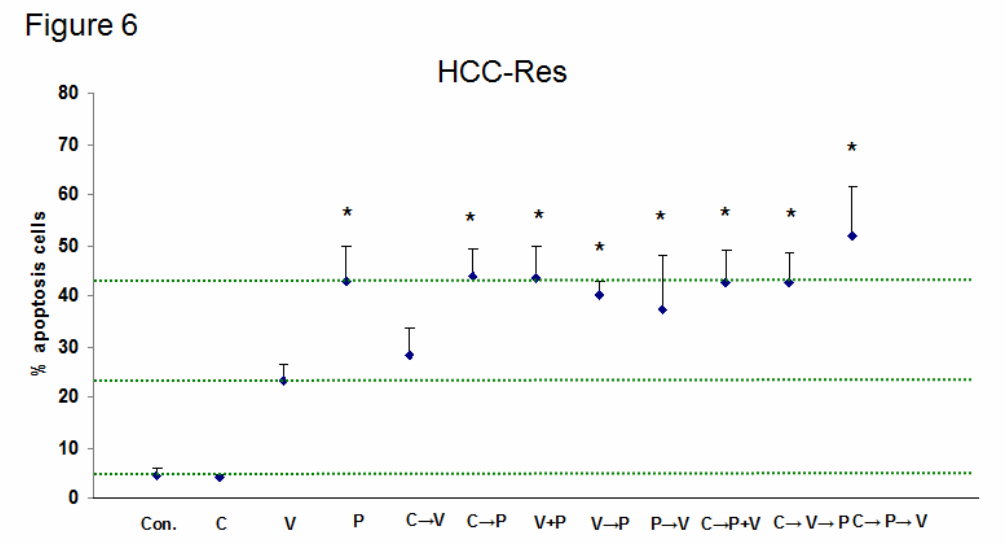


Figure 6. Effect of vinorelbine, pemetrexed and different chemotherapy schedule on apoptosis in HCC-res cells: apoptosis was analysed after the cells were treated with different schedule of combination of cisplatin, vinorelbine, and pemetrexed respectively for 24 h. The percentages of apoptotic cells in all treated groups except vinorelbine group were significantly higher than the control group. (n=3, * = $P<0.05$ versus other experimental groups)

3.4 Effect of vinorelbine, pemetrexed and different chemotherapy schedule on cytoplasma Ca^{2+}

Different therapy schedule has been applied on HCC and HCC-res cells with or without cisplatin treatment. Cytoplasm free calcium concentration was measured after 48 h.

In HCC cells, cytoplasm free calcium concentration in all treated groups except vinorelbine treated group was significantly elevated than that of the control group. Single drug exposure of vinorelbine and pemetrexed resulted in cytoplasm free calcium concentration of 176.52±86.23 nM and 265.28±67.39 nM respectively.

Sequential double drug application of Cis→Vin or Cis→Pem resulted in cytoplasm free calcium concentration of 256.25±57.86 nM and 377.11±112.81 nM respectively. Simultaneous exposure of Vin+Pem resulted in cytoplasm free calcium concentration of 261.58±68.45 nM while sequential application of Vin→Pem or Pem→Vin as 368.42±92.20 nM and 277.06±48.39 nM respectively.

In the schemes of triple drug treatments, the highest cytoplasm free calcium concentration was found in Cis→Pem→Vin treated group as 694.05±170.15 nM. (n=3) (Figure 7)

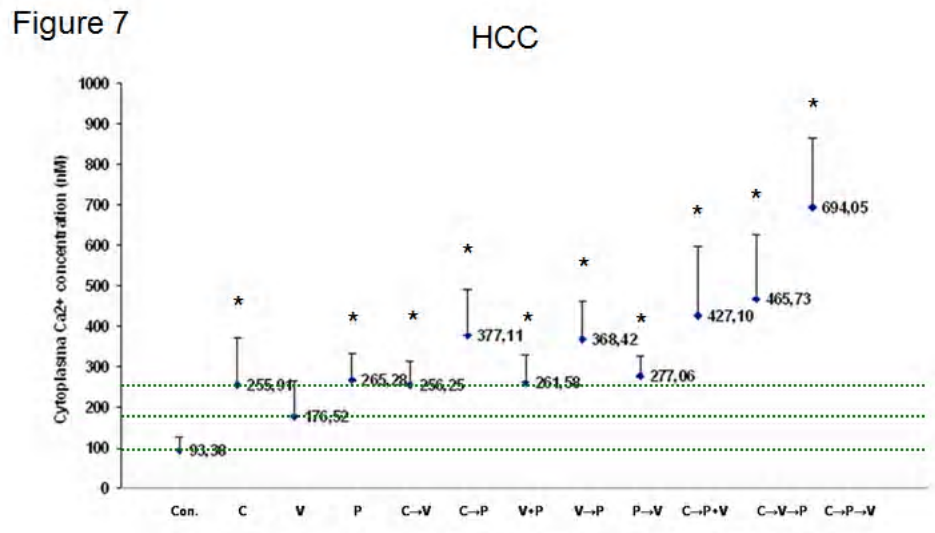


Figure 7. Effect of vinorelbine, pemetrexed and different chemotherapy schedule on cytoplasm free calcium in HCC cells: Different therapy schedule has been applied on HCC cells with or without cisplatin treatment. Cytoplasm free calcium concentration was measured after 48 h. Cytoplasm free calcium concentration in all treated groups except vinorelbine group was significantly elevated than that of the control group. (n=3, * = P<0.05 versus control group)

In the cisplatin resistant HCC-res cells, 1 µM cispatin failed to induce a cytoplasm free calcium concentration increase. In HCC-res cells, cytoplasm free calcium concentration in all other treated groups except vinorelbine treated group was significantly elevated than that of the control group.

Single drug exposure of vinorelbine and pemetrexed resulted in cytoplasm free calcium concentration of 149.48±38.88 nM and 304.77±78.48 nM respectively.

Sequential double drug application of Cis→Vin or Cis→Pem resulted in cytoplasm free calcium concentration of 152.14±42.04 nM and 357.64±42.83 nM respectively. Simultaneous exposure of Vin+Pem resulted in cytoplasm free calcium concentration of 274.56±86.14 nM while sequential application Vin→Pem or Pem→Vin as 359.85±73.65 nM and 266.32±58.36 nM respectively.

In the schemes of triple drug treatments, the highest cytoplasm free calcium concentration was found in Cis→Pem→Vin as 391.19±76.58 nM, which was significantly lower than that of treated HCC cells. (n=3) (Figure 8)

Figure 8

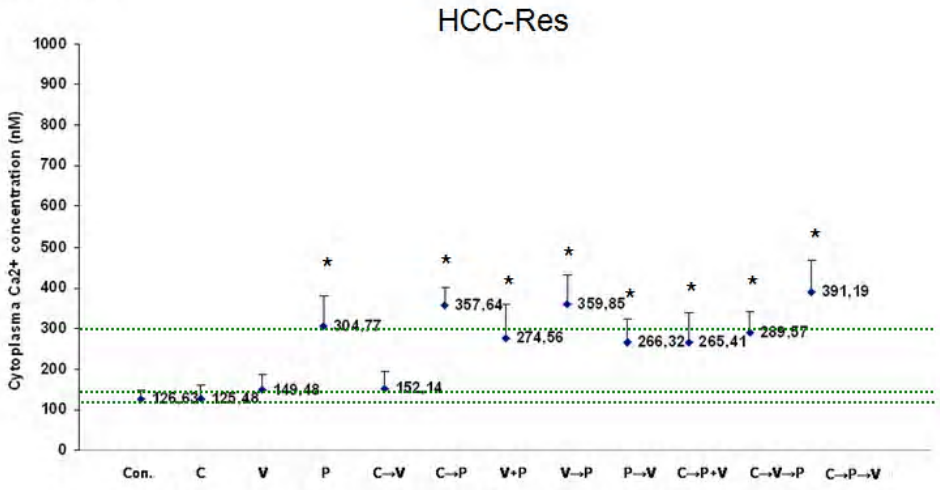


Figure 8. Effect of vinorelbine, pemetrexed and different chemotherapy schedule on cytoplasm calcium in HCC-res cells: Different therapy schedule has been applied on HCC-res cells with or without cisplatin treatment. Cytoplasm free calcium concentration was measured after 48 h. Cytoplasm free calcium concentration in all treated groups except cisplatin and vinorelbine treated groups was significantly elevated than that of the control group. (n=3, * = $P<0.05$ versus control group)

4 DISCUSSION

4.1 Schedule-dependent cytotoxic synergism in lung cancer cell lines

Mehta *et. al.* examined chemotherapeutic combination in breast cancer and found that the median time to disease progression and survival was significantly shorter for patients treated with any combination of agents, exhibiting either extreme or intermediate in vitro drug resistance, in comparison with patients having tumours with low in vitro resistance to both drugs [88]. In order to investigate the different effect of therapeutic schedules in adeno lung cancer cells, different schemes with vinorelbine and pemetrexed with or without cisplatin were applied on adeno lung carcinoma HCC and cisplatin resistant adeno lung carcinoma HCC-res cells.

HCC and HCC-res cells have shown sensitivity to both pemetrexed and vinorelbine. Pemetrexed has shown cytotoxic activity in several human cell lines, including lung carcinoma cell lines, which agrees with its cell growth inhibitory effect on HCC cell line.

Animal studies have shown that vinorelbine has favourable antitumor activity on lung cancer cells and can induce cellular differentiation. Recently, much interest has been focused on the therapeutic efficacy of vinorelbine in NSCLC. Clinical studies demonstrated that vinorelbine has antitumor activity in patients with advanced NSCLC as a single agent and in combination with other anticancer agents including cisplatin or gemcitabine. Moreover, vinorelbine has been proven to improve the patient's QOL as well as prolong survival of elderly NSCLC patients with poor performance status [89].

The therapeutic scheme as sequential application of pemetrexed and vinorelbine with or without cisplatin has shown a synergistic effect in cell growth inhibition, while simultaneous exposure of pemetrexed and vinorelbine and converse application sequence, with or without cisplatin, did not provide antagonistic or additive effect in cell growth in both HCC and HCC-res cells. Pemetrexed has been found to induce synchronization of cell cycle events. For instance, it was shown in CCRF-CEM lymphocytic leukemia cells that apoptosis was preceded by accumulation at the G1/S interface within 12 h of pemetrexed application, after which the cell population entered S phase synchronously. Cellular accumulation in the S phase may sensitize cells to the cytotoxic effects of other chemotherapeutic drugs. In colon carcinoma cell line, the cytotoxicity of gemcitabine was increased significantly with

pemetrexed added 24 h previously [20]. Additional synergistic cytotoxicity has been demonstrated with pemetrexed in combination with other chemotherapeutics agents in NSCLC in vitro and in vivo, including cisplatin, carboplatin, and oxaliplatin. The effect of radiotherapy was also found to be enhanced with pemetrexed pre-application. Administration of pemetrexed prior to fractionated radiation therapy delayed tumor growth in mice with NSCLC xenografts. Schedule dependent cytotoxic effect in NSCLC cell lines has been shown with pemetrexed in combination with erlotinib. Synergism was most pronounced with exposure to pemetrexed plus erlotinib for 24 h followed by erlotinib for 48 h [90].

Dan Zhang *et al.* has investigated in pemetrexed-resistant cell lines, and shown that the cells maintained sensitivity to 5-fluorouracil, docetaxel, SN-38, and vinorelbin; however, all of the cell lines showed cross-resistance to cisplatin [91].

4.2 Effect of pemetrexed, vinorelbine and combined chemotherapy schedules on the calcium homeostasis and apoptosis in lung cancer cell lines

Squamous cell lung carcinomas origin from metaplastic bronchial epithelium and small cell lung carcinomas are believed to origin from neuro-epithelial bodies. But the origin of large cell carcinomas and adeno carcinomas is less clear [86]. It has been known for a long time that calcium signals govern a host of vital cell functions and so are necessary for cell survival. More recently, it has become more clear that cellular calcium overload or perturbation of intracellular calcium compartmentalization can cause cytotoxicity and trigger apoptosis or necrotic cell death [92]. Ca^{2+}-dependent processes are involved with the caspases, the mainstream apoptosis executioners, and the interfering with the sequestration of calcium into intracellular pools as ER can be sufficient to trigger apoptosis as part of a stress response [93]. The earliest link that was found between calcium and apoptosis was that calcium induced a typical apoptosis ladder-like DNA fragmentation pattern in isolated thymocytes nuclei through the activation of a Ca^{2+} -and Mg^{2+} -dependent endonuclease [94]. It has been discovered in mouse T-lymphocytes that high-dose pharmacologically raising cytoplasmic calcium resulted in apoptosis but low-dose treatment gave a resistant capacity to apoptosis [95]. It has been demonstrated that increased cytoplasm free calcium concentration is involved in number of cellular event, including apoptotic pathways. The elevation of cytoplasm calcium concentration might be the result of calcium entering from extracellular

space or calcium being released from intracellular calcium stores. In apoptosis, it results in an increase in the mitochondrial calcium concentration, which opens the permeability transition pore followed by an efflux of cytochrome C. As a consequence, cytochrome C amplifies the calcium release from the ER and activates the intrinsic apoptotic pathway via caspase 9 [96].

The sequential application of pemetrexed and vinorelbine with or without cisplatin has shown a synergistic effect to increase cytoplasm calcium concentration and apoptosis, which confirmed the relationship between increase of cytoplasm calcium and apoptosis by cytotoxic agents. Recent advances in elucidating the molecular biology of lung carcinogenesis have elucidated novel drug targets, and treatments are rapidly evolving into specialized agents that hone in on specific aspects of the disease. Of particular interest is blocking tumor growth by targeting the physiological processes surrounding angiogenesis, pro-tumorigenic growth factor activation, anti-apoptotic cascades and other cancer-promoting signal transduction events. The effect of the chemotherapy compounds and combination of these on calcium and apoptosis would light up a new perspective in cancer therapy and mechanism of tumorigenesis.

4.3 Outlook

Worldwide, lung cancer is the most common cancer in terms of both incidence and mortality with the highest rates in Europe and North America. First-line chemotherapy often leads to encouraging responses in lung cancer, but during the treatment process, resistance to the chemotherapy frequently occurs and ultimately limits the life expectancy of the patient. The combination of novel chemotherapeutic schedules might be a solution other than new drug development.

Therefore, the incorporation of new combination of vinorelbin, pemetrexed with cisplatin into the therapeutic regimens of adenocarcinoma of the lung casts light into the cancer therapy field. A new therapy strategy could be to combine the chemotherapy medication with consideration of different cytotoxic mechanisms, in order to destruct tumour. Clinical trials could verify these findings.

As the therapeutic combination of vinorelbin, pemetrexed and cisplatin has not been investigated in adeno carcinoma, although it has been utilized in NSCLC treatment and

showed promising effect. My findings support the further clinical usage of the chemotherapy schemes in lung cancer therapy. The combination with targeted therapy, anti-oncogenic molecules as Hedgehog inhibitors, anti-RAS, AKT, or MEK molecules could be also beneficial to tumors with more than oncogenic mechanisms involved. A new class of therapeutic agents as combination partner could be investigated in the future.

Moreover, the further research into cytotoxic mechanism in lung cancer and interaction between different chemotherapeutics still needs to be done, for instance the molecular changes and the cross talk between oncogenic pathways. Since it has been found in our work that vinorelbin, pemetrexed, and combination of different schedules influences the homeostasis of calcium and apoptosis, as the universe second messenger, the effect of cytotoxic agents on calcium signalling was shown. To understand and utilize distinct comprehension of calcium signalling and chemotherapy is necessary for the furthermore set up of new cancer therapy methods.

5 CONCLUSION

In this study, vinorelbine and pemetrexed caused a strong dose-dependent cytotoxic effect in both HCC cells (adenocarcinoma of the lung) and HCC-res cells (resistant against cisplatin). The IC_{50} values of vinorelbine against HCC and HCC-res cells were 10.34±1.12 nM and 9.98±2.12 nM, respectively. The IC_{50} values of pemetrexed against these cells were 110.77±17.28 nM and 118.89±18.77 nM respectively.

The application of different therapy schedule including pemetrexed and vinorelbine with or without cisplatin treatment induced a significant time dependent cell growth inhibition on HCC naïve and cisplatin resistant cells. The therapy scheme of sequential application of Cis→Pem→Vin showed the strongest inhibitory effect on both HCC and HCC-res cells.

The application of different therapy schedules including pemetrexed and vinorelbine with or without cisplatin treatment on HCC naïve and cisplatin resistant cells increased the percentage of cells undergoing apoptosis, except application of vinorelbine alone. In both HCC and HCC-res cells, sequential application of Cis→Pem→Vin was found the most effective to induce apoptosis.

The application of different therapy schedules including pemetrexed and vinorelbine with or without cisplatin treatment on HCC naïve and cisplatin resistant cells increased cytoplasm calcium concentration except application of vinorelbine alone failed to increase cytoplasm calcium concentration in HCC cells. The most elevated cytoplasm calcium concentration was found in the cells treated with sequential application of Cis→Pem→Vin in both HCC and HCC-res cells. The calcium overload could lead to apoptosis, which was related to the cell growth inhibitory effect of chemotherapeutics in lung cancer cells.

Sequential application of cisplatin, pemetrexed and vinorelbine has shown a synergistic effect in cell growth inhibition, apoptosis induction, and cytoplasm calcium concentration elevation in adeno lung carcinoma cells and also cisplatin resistant adeno lung carcinoma cells.

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APPENDIX I – LIST OF FIGURES

APPENDIX II - LIST OF ABBREVIATIONS

NSCLC: non-small cell lung cancer

SCLC: small cell lung cancer

Cis: cisplatin

Pem: pemetrexed

Vin: vinorelbine

EGFR: epidermal growth factor receptor

SNP: single-nucleotide polymorphisms

NLST: National Lung Screening Trial

QOL: quality of life

MSC: mesenchymal stromal cells

CAFs: carcinoma-associated fibroblasts

TAM: Tumor-associated macrophages

PPARγ: peroxisome proliferator-activated receptor-γ

Treg: regulatory T cells

ABC transporter: ATP-binding cassette transporter

MDR: multidrug resistance

Top: topoisomerase

ECM: extracelluar matrix

MMR: mismatch repair

MMP: matrix metalloproteinase

PDGFR: platelet-derived growth factor receptor

DISC: death inducing signal complex

PFS: progression-free survival

VEGF: vascular endothelial growth factor

FGFβ: fibroblast growth factor β

TNFα: tumor necrosis factor α

TS: thymidylate synthase

AICARFT: aminoimidazolecarboxamide ribonucleotide formyltransferase

dUMP: fluorodeoxyuridine monophosphate

dTMP: deoxythymidine monophosphate

FPGS: folylpolyglutamate synthetase

ER: endoplasmic reticulum

SR: sarcoplasmic reticulum

$[Ca^{2+}]_c$: cytoplasm free Ca^{2+}

$[Ca^{2+}]_{ER}$: endoplasmic reticulum Ca^{2+}

SOCC: store-operated Ca^{2+} channel

VOCC: voltage-operated Ca^{2+} channel

ROCC: receptor-operated Ca^{2+} channel

IP3: inositol 1, 4, 5-trisphosphate

IP3R: inositol 1, 4, 5-trisphosphate receptor

RyR: ryanodine receptor

SERCA: sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase

PARP1: poly ADP-ribose polymerase-1

ROI: regions of interest

CPA: cyclopiazonic acid

Kd: dissociation constant

HCC-res: HCC cisplatin resistant cell

ROI: region of interest

APPENDIX III - CURRICULUM VITAE

ZHE WANG

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EDUCATION

Feb.2012- now	LMU, PhD candidate in Medicine
Sep.2007- Jul.2010	Harbin Medicine University, Master in Surgery
Sep.1999- Jul.2004	Qiqihar Medical University, Bachelor in Medicine

LANGUAGE SKILLS

English	Fluent written and verbal skills
Chinese	Mother tongue

WORKING EXPERIENCE

Aug. 2004-Jan. 2011	Surgeon	Heilongjiang Nongken Hospital, Thorax Surgery Department

RESEARCH EXPERIENCE

2007-2010

1. Efficacy of spraying of video-assisted thoracoscopic talc and combined thermotherapy of the malignant pleural effusion patients.
2. Application of ultrasonic scalpel in video-assisted thorascopic lymphadenectomy.
3. Investigation of value of serum endostatin level in early diagnosis of lung cancer.

2011-now

Schedule-dependent interactions between pemetrexed and vinorelbine in human lung cancer cells.

Eidesstattliche Versicherung

Zhe WANG

Name, Vorname

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

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