Pathogenesis of neurotoxicity and role of signaling in the efficacy of anticancer agents

Abolfazl Avan

A flower can grow even in a hostile desert!
With the optimal treatment,
the cancer patient can also live a happy life!
The studies described in the present thesis were performed at the Laboratory of Medical Oncology, Department of Medical Oncology, VU University Medical Center Amsterdam, Amsterdam, the Netherlands.
Pathogenesis of neurotoxicity and role of signaling in the efficacy of anticancer agents

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door

Abolfazl Avan
geboren te Mashhad, Iran
promotor: prof.dr. G.J. Peters
copromotor: dr. E. Giovannetti
Members of the reading committee:

dr. J. Buter
dr. T.U. Hoogenraad
dr. J.R. Kroep
dr. T.J. Postma
prof.dr. E.F. Smit
prof.dr. T. Würdinger
Human beings are members of a whole,
In creation of one essence and soul.
If one member is afflicted with pain,
Other members uneasy will remain.
If you've no sympathy for human pain,
The name of human you cannot retain!

Iranian poet Sa'adi

... whoever saves a soul, it is as if he had saved entire mankind ... 
Quran, (chapter 5: verse 32)
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CHAPTER 1

Introduction and Outline
Neuropathy: an intricate consequence of platinum agents

1. General overview

Peripheral neuropathy due to chemotherapy is one of the major dose-limiting side effects of many anticancer medications and their characteristics are often related to both the choice of anticancer drugs and the cumulative doses [1]. Since peripheral nervous system is not protected as efficiently as the central nervous system from exogenous agents, it is more sensitive to the toxic action. The overall incidence of chemotherapy-induced peripheral neuropathy is estimated to be roughly 38%, which may vary depending on type and duration of treatment as well as the assessment tool [2;3]. Higher risk and incidence of this side effect is reported with platinum compounds, vinca alkaloids, bortezomib, and/or taxanes amongst others [2;4].

New insights in the understanding of the pathophysiology, pathogenesis, and individual susceptibility factors may pave the way for the development of new prophylactic and therapeutic measures. The pathophysiology and pathogenesis of platinum-induced peripheral neurotoxicity has been reviewed as well as the potency of different neurotropic and neuroprotective drugs (chapter 2). These data may help to design novel models to develop alternative options in the treatment of platinum-induced neuropathy.

Among more than 3000 platinum compounds, cisplatin, carboplatin, and oxaliplatin are the most commonly used in the clinic, while other analogs of platinum, including nedaplatin, lobaplatin, and heptaplatin are only approved in Japan, China, and South Korea, respectively [5]. The antitumor effect of platinum compounds is mediated via the formation of DNA-platinum adducts, which may activate signaling pathways and apoptosis. This process may induce neurotoxicity.

1.2. History

In 1965, Rosenberg serendipitously discovered cisplatin while evaluating the effect of electric fields on the cell division of bacteria [6]. Later, in 1969, its antineoplastic characteristics were identified [7], and in the early 70s, it was applied in clinical treatment. Platinum compounds have been increasingly used in routine oncological clinical practice, and are currently the mainstay of cancer treatment. The U.S. Food and Drug Administration approved cisplatin for use in testicular and ovarian cancers in 1978 and in the UK (and in several other European countries) in 1979. Insight into mechanisms of antitumor action for cisplatin led to the design of novel platinum analogs with better pharmacological profiles and safety, such as carboplatin and oxaliplatin [5]. Oxaliplatin received European approval in 1996 and approval by the U.S. Food and Drug Administration in 2002. While the toxicity profile differs among platinum agents, peripheral neuropathy is a common feature, particularly with cisplatin and oxaliplatin.

1.3. Pathophysiology

Peripheral neurotoxicity of platinum compounds is related to several molecular mechanisms, including dorsal root ganglia cytotoxic inflammatory changes, mitotoxicity, enhanced oxidative stress, voltage-gated ion (sodium/potassium/calcium) channel dysfunction, functional impairment of ion channels of the transient receptor potential family, induction of neuronal apoptosis in dorsal root ganglia, and demyelination [8]. Accumulation of platinum products mainly in the dorsal root ganglia, as well as peripheral neurons may provoke chronic neuropathy. Since these neurons are post-mitotic and not dividing, the formation of DNA adducts is not lethal, but results in DNA-strand breaks. The extent of DNA crosslinks in dorsal root ganglia at specific cumulative dose corresponds to the degree of neurotoxicity [9]. Cisplatin produces more adducts in the dorsal root ganglia compared to oxaliplatin, which may explain its higher neurotoxicity. Platinum adducts may also affect neurons, while brain and spinal cord are protected by the blood brain barrier [9].
1.4. Neuroprotection and Treatment

Several model systems have been used to study the nature of overall neurotoxicity and the effect of potential neuroprotective drugs. There are heterogeneous results on the efficacy of neuroprotective agents (i.e. detoxicants, nerve growth factor stimulants, antioxidants, electrolytes, chelators, ion channel modulators, etc.), but dose adjustment and/or drug withdrawal seem to be the most effective and commonly used therapeutic against platinum-induced peripheral neurotoxicity. In addition, different antidepressants, anticonvulsants, and some other compounds have been tested against platinum-induced peripheral neurotoxicity (chapter 2, 3, and 4); however, only duloxetine is recommended for therapeutic purposes.

2. Characteristics of Platinum analogs

Cisplatin, carboplatin, and oxaliplatin are nowadays in routine clinical use. The characteristics of these three platinum compounds and a novel oral analog are discussed in the next sections.

2.1. Cisplatin

Cisplatin, cis-diaminedichloroplatinum (Figure 1.1) is a heavy metal complex containing a central atom of platinum surrounded by two chloride atoms and two ammonia molecules in the cis position. As first line choice of treatment, cisplatin is approved against metastatic testicular tumors, ovarian carcinoma, advanced bladder cancer, and frequently used, often in combination, against head and neck squamous cell cancer, cancers of esophagus, malignant pleural mesothelioma, non-small cell lung cancer, cervix, endometrium, and osteogenic sarcomas. Cisplatin is highly mutagenic and carcinogenic in both in vitro and in vivo models [10]. The antitumor properties of cisplatin are ascribed to the kinetics of its chloride ligand displacement reactions leading to DNA crosslinks. This causes DNA bending, which leads to impeding of DNA replication, transcription and other nuclear functions, which finally interferes with cancer cell proliferation and tumor growth [5,11]. The nucleotide excision repair, e.g. ERCC-1, is the principal repair pathway for removal of cisplatin-adducts [12-14], but may also involve recognition of the damage by High Mobility Group (HMG), nonhistone proteins, and mismatch repair proteins [15]. Accordingly, DNA repair produces resistance to cisplatin through the failure to recognize the cisplatin-DNA adduct and propagates a signal to the apoptotic machinery.

A number of additional properties of cisplatin are now emerging, which confer drug resistance, including activation of several signal transduction pathways, such as Akt, ABL, p53, and MAPK/JNK/ERK leading to apoptosis [16]. Cisplatin may activate or inhibit these pathways through alteration of receptor or lipid molecules in the cell membrane, regulation of protein kinases, or activation of the DNA repair pathways after DNA damage (reviewed in [5]). Cisplatin resistance could also arise from decreased tumor blood flow, reduced platinum uptake, increased efflux, intracellular detoxification, e.g. by glutathione, decreased binding (due to high intracellular pH), DNA repair, decreased mismatch repair, defective apoptosis, antiapoptotic factors, modulation of signaling pathways, or presence of quiescent non-cycling cells [17]. Extracellular environment and diet may also influence cisplatin efficacy, as high extracellular PH [18,19], presence of extracellular matrix proteins fibronectin, type IV collagen and laminin [20], extracellular gamma-glutamyltransferase [21], and bicarbonate intake [22] may decrease the intake cisplatin by tumor cells.
Some therapeutic interventions at different molecular levels have been identified to sensitize the cancer cells to cisplatin (chapter 3). The discovery of novel platinum molecules with higher specificity for the targets and better safety profile could also lead to a breakthrough in bypassing cisplatin resistance [23].

2.2. Carboplatin

Carboplatin (Figure 1.2) was introduced in the 1980s, following a joint drug development and screening process between industry and academia. It has markedly less toxicity to the kidneys and nervous system at conventional doses than cisplatin and causes less gastrointestinal problems, while retaining comparable antineoplastic activity, particularly in ovarian cancers [2]. Carboplatin resistance could arise by similar mechanisms as cisplatin [17]. Nevertheless, the most recent Cochrane review comparing the toxicity of carboplatin versus cisplatin in combination with third-generation drugs for advanced non-small cell lung cancer reported an almost two-times higher rate of neurotoxicity in the carboplatin group [24].

2.3. Oxaliplatin

Oxaliplatin, (trans-R,R-1,2-diaminocyclohexane) oxalate platinum (II) (Figure 1.3), is a third generation platinum drug with a diaminocyclohexane (DACH) entity, which is approved for the clinical treatment of colon cancer. Oxaliplatin produces the same type of inter- and 1,2-GG intrastrand cross-links as cisplatin, but has a spectrum of activity and mechanisms of action and resistance different from those of cisplatin and carboplatin [5], suggesting a different mechanism of action. The cellular and molecular aspects of the mechanism of action of oxaliplatin are not yet completely elucidated.

The pattern of oxaliplatin side effects, include neurotoxicity, hematological toxicity, and gastrointestinal tract toxicity correlated with the cumulative-dose of oxaliplatin [25]. The neurological manifestations of oxaliplatin-induced peripheral neuropathy may vary between a subacute transient peripheral sensory neuropathy (i.e. paresthesias and dysesthesia in the extremities sometimes accompanied by muscular cramps) and a late-onset neuropathy (i.e. deep sensory loss, ataxia, and functional impairment). The peripheral neuropathy of oxaliplatin might be caused by its ability to decrease both Na+ and K+ currents and thus modifying the voltage-dependent ionic channels mainly by altering the external surface membrane potential [26].

Compared to cisplatin and carboplatin, oxaliplatin is approved for a narrower spectrum of cancers, including non-small cell lung cancer and colorectal cancers, and it is usually administered in combination with other anticancer agents, such as 5-fluorouracil, gemcitabine, topoisomerase inhibitors, and taxanes [27;28].

2.4. Satraplatin

Satraplatin (JM216; Figure 1.4) is the first oral platinum analog [29] having greater lipophilicity compared to carboplatin and cisplatin [30], favorable bioavailability [29;31], and effective penetration through blood brain barrier comparable to that of carboplatin and cisplatin [30] plus a good safety profile (lacking nephrotoxicity and neurotoxicity) compared to cisplatin in vivo [32;33]. Some clinical studies have shown the efficacy of satraplatin in chemonaive small-cell lung cancer and advanced non-
small cell lung cancer [34;35], as well as cervical cancer, hormone refractory prostate cancer, and breast cancer [36-38].

3. Etiology of platinum-induced neurotoxicity and resistance

3.1. Polymorphisms and modulation of signaling

Association of polymorphisms in some genes with platinum-induced peripheral neuropathy is a matter of debate. There are controversial reports about some genes that may contribute to the incidence and/or severity of neuropathy in patients being treated with platinum analogues (chapter 2). The genes, include ATP-binding cassette sub-family B member 1 (ABCB1), ATP-binding cassette sub-family B member 1 or 2 (ABCC1, C2 or CG2), alanine-glyoxylate aminotransferase (AGXT), cyclin H (CCNH), catechol O-methyltransferase (COMT), excision repair cross-complementation group 1 (ERCC1) and ERCC2 (alias XPD, Xeroderma-Pigmentosum group-D), integrin beta 3 (ITGβ3), glutathione S-transferases (e.g. GSTM1, GSTM3 and GSTT1), voltage-gated sodium channel genes (SCNAs), thiopurine S-methyltransferase (TPMT), and X-ray repair cross-complementing protein 1 (XRCC1). Although some data support the role of the mentioned genetic variations in the presentation and severity of platinum-induced peripheral neurotoxicity, the heterogeneity of the results diverse (chapter 2).

3.2. Role of signaling in treatment efficacy

The Phosphatidylinositol 3/protein kinase B (PI3K/Akt) signal transduction pathway controls most hallmarks of cancer, including metabolism, cell survival, cell cycle progression or regulation of apoptosis, protein synthesis, motility and genomic instability by phosphorylation of the substrates (chapters 5-10). Aberrant activation of this pathway has been associated with the development of cancer. The Akt pathway serves as a survival pathway, meaning that its activation inhibits major steps in cell death regulation and directly stimulates other survival pathways. Moreover, consecutive evidence affirms overactivation of PI3K/Akt pathway in nearly one third of carcinomas. Given the promotion of the malignant phenotype and implication of drug resistance due to excessive activation of Akt cascade, modulation of this survival pathway may enhance the efficacy of chemoradiotherapy. Accordingly, different Akt inhibitors, e.g. perifosine and MK-2206, or PI3K modulators, e.g. LY294002 and wortmannin, have been used in numerous preclinical and clinical studies to enhance the cytotoxic effects of the DNA-targeted treatments to sensitize the tumor to the anticancer agent (chapters 5, 6).

3.3. Repair enzymes and platinum efficacy

Antineoplastic activity of platinum compounds is mainly mediated via the formation of toxic platinum-DNA adducts. Removal of these adducts, as the normal protective mechanism, causes chemoresistance. Nucleotide excision repair and mismatch repair system are the principal repair pathways for removal of platinum-DNA adducts. Enzymes involved in the recognition of DNA damage, unwinding, subsequent excision of the damaged nucleotides, and insertion of new deoxynucleoside triphosphates into the DNA are Transcription Factor II H, Xeroderma Pigmentosum C, D, F enzymes, excision-repair cross-complementing group 1, DNA polymerases δ and ε, and ligase 1. Single nucleotide polymorphisms in any of these genes may affect the repair capacity and contribute to individual variations in chemotherapy response (chapters 7-10).
5. Outline of the thesis

Part 1. Drug-induced Neurotoxicity: Pathogenesis and Treatment

5.1. Chapter 2

Neurotoxicity is an undesirable consequence of platinum-based chemotherapy, which prevents administration of the full efficacious dosage. It often leads to treatment withdrawal, affects the patients’ quality of life, and sometimes is irreversible. Chapter 2 discusses available preclinical and clinical evidence of the pathogenesis and pathophysiology of platinum-induced peripheral neurotoxicity as well as available neuroprotective and therapeutic strategies to avert this side effect. These data may help to improve or develop alternative options in the treatment of platinum-induced neuropathy, along with in vitro models, and appropriate trials planning to find the best patient-oriented solution.

5.2. Chapter 3

Different models have been introduced to study chemotherapy-induced neurotoxicity. Chapter 3 describes a method for evaluation of the neurotoxicity using the neurite outgrowth in PC12 rat pheochromocytoma cells. With this method, we investigated the neurotoxicity of oxaliplatin, bortezomib, and epothilone-B, and tested the potential neuroprotection of amifostine. We also investigated the suitability of two markers of neuronal differentiation, cyclin-B2 and BIRC5.

5.3. Chapter 4

Calcium/magnesium infusion is one of the popular preventive strategies against oxaliplatin-induced neurotoxicity. Chapter 4 discusses whether or not current evidence supports its efficacy.

Part 2. Signaling and the Efficacy of Cancer Treatment

5.4. Chapter 5

Activation of the Akt-survival pathway is a mechanism of resistance to DNA-targeted drugs. Chapter 5 reviews the effect of common anticancer drugs, i.e. platinum agents, taxanes, antimetabolites, and tumor antibiotics on Akt pathway, and whether Akt inhibitors may enhance the cytotoxic effects of the DNA-targeted treatments by antagonizing the Akt survival pathway and sensitizing tumors to anticancer agent.

5.5. Chapter 6

There is increasing evidence of a constitutive activation of Akt in pancreatic cancer. Chapter 6 discusses the therapeutic potential of the novel Akt inhibitor perifosine in combination with gemcitabine in pancreatic ductal adenocarcinoma cells, showing that perifosine can interfere with cell proliferation, induce apoptosis, reduce migration/invasion, and synergistically interact with gemcitabine in cells with phospho-Akt overexpression.

5.6. Chapter 7

Increasing the formation and retention of platinum-DNA adducts by decreasing the DNA-repair enzymes may boost the antineoplastic effects of the treatment and overall survival. Chapter 7 explores the importance of protein/mRNA expression-analysis, as well as the role of polymorphism in the DNA repair enzymes in non-small cell lung cancer and pancreatic ductal adenocarcinoma.

5.7. Chapter 8

Satraplatin is the only oral analog among platinum agents. Chapter 8 discusses the potential synergism between erlotinib, an epidermal growth factor receptor (EGFR) inhibitor and JM118, the active metabolite of satraplatin. For this purpose, seven cancer cell lines were examined for the expression of
Akt, Erk and p38, and cell cycle proteins, as well as cell cycle distribution, cell death, and adduct formation.

5.8. Chapter 9

Single nucleotide polymorphisms may be indicative of a shorter survival. Chapter 9 explores the role of novel predictive biomarkers for cachexia, as a direct cause of reduced quality of life and shorter survival, in pancreatic ductal adenocarcinoma. SELP and AKT1 polymorphisms may be predictive of the risk of cachexia and death in this type of cancer.

5.9. Chapter 10


5.10. Chapter 11

Chapter 11 discusses the results presented in this dissertation.

References


CHAPTER 2

Platinum-Induced Neurotoxicity and Preventive Strategies: Past, Present, and Future

Abolfazl Avan,¹ Tjeerd J. Postma,² Cecilia Ceresa,³ Amir Avan,¹ ⁴ Guido Cavaletti,³ Elisa Giovannetti,¹ Godefridus J. Peters¹*

Departments of ¹Medical Oncology and ²Neurology, VU University Medical Center, Amsterdam, The Netherlands; ³Department of Surgery and Interdisciplinary Medicine, University of Milano-Bicocca, Monza, Italy; ⁴Department of New Sciences and Technology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

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Platinum-induced neurotoxicity and preventive strategies: past, present, and future

ABSTRACT: Neurotoxicity is a burdensome side effect of platinum-based chemotherapy that prevents administration of the full efficacious dosage and often leads to treatment withdrawal. Peripheral sensory neurotoxicity varies from paresthesia in fingers to ataxic gait, which might be transient or irreversible. Because the number of patients being treated with these neurotoxic agents is still increasing, the need for understanding the pathogenesis of this dramatic side effect is critical. Platinum derivatives, such as cisplatin and carboplatin, harm mainly peripheral nerves and dorsal root ganglia neurons, possibly because of progressive DNA-adduct accumulation and inhibition of DNA repair pathways (e.g., extracellular signal-regulated kinase 1/2, c-Jun N-terminal kinase/stress-activated protein kinase, and p38 mitogen-activated protein kinase), which finally mediate apoptosis. Oxaliplatin, with a completely different pharmacokinetic profile, may also alter calcium-sensitive voltage-gated sodium channel kinetics through a calcium ion immobilization by oxalate residue as a calcium chelator and cause acute neurotoxicity. Polymorphisms in several genes, such as voltage-gated sodium channel genes or genes affecting the activity of pivotal metal transporters (e.g., organic cation transporters, organic cation/carnitine transporters, and some metal transporters, such as the copper transporters, and multidrug resistance-associated proteins), can also influence drug neurotoxicity and treatment response. However, most pharmacogenetics studies need to be elucidated by robust evidence. There are supportive reports about the effectiveness of several neuroprotective agents (e.g., vitamin E, glutathione, amifostine, xaliproden, and venlafaxine), but dose adjustment and/or drug withdrawal seem to be the most frequently used methods in the management of platinum-induced peripheral neurotoxicity. To develop alternative options in the treatment of platinum-induced neuropathy, studies on in vitro models and appropriate trials planning should be integrated into the future design of neuroprotective strategies to find the best patient-oriented solution.

Implications for Practice: Neurotoxicity is a burdensome side effect of platinum-based chemotherapy that prevents administration of the full efficacious dosage and often leads to treatment withdrawal. This review summarizes preclinical and clinical evidence of pathogenesis and pathophysiology of platinum-induced peripheral neurotoxicity, as well as available evidence of neuroprotective and therapeutic strategies. These data may help to develop alternative options in the treatment of platinum-induced neuropathy, studies on in vitro models, and appropriate trials planning to find the best patient-oriented solution.

Introduction

Since the discovery of cisplatin in the mid-1960s, many platinum compounds (more than 3,000 compounds) have been developed. Thirty-five of these compounds have exhibited adequate pharmacological advantages (e.g., reaching sufficiently high plasma levels not associated with common toxicities, such as renal toxicity and thrombocytopenia) [1]. Some of them have been registered or are being considered for registration for treatment of different cancers, such as the second (carboplatin, nedaplatin, tetraplatin, and iproplatin) and third (oxaliplatin, lobaplatin, heptaplatin, satraplatin, and LA-12) generation, usually with better safety profiles [2–4].

Despite the efficacy of platinum analogs in cancer treatment, serious side effects, especially peripheral sensory neurotoxicity, often at their full efficacious doses or may considerably affect the quality of life of cancer patients being treated with them [5, 6]. Cisplatin was the first heavy metal used in several kinds of solid tumors, including lung, ovary, testis, bladder, head and neck, and endometrium [7, 8]; most patients develop a symptomatic neuropathy [9]. Second and third generations of platinum compounds have emerged in attempts to reduce the toxicity of cisplatin. Carboplatin, a second generation of platinums used to treat ovarian, non-small cell lung, and refractory testicular cancers, was thought to be associated with a lower risk of developing neurotoxicity [9]. However, the most recent Cochrane review comparing the toxicity of carboplatin versus cisplatin in
combination with third-generation drugs for advanced non-small cell lung cancer reported an almost two times higher rate of neurotoxicity in the carboplatin group [10]. Oxaliplatin, as a widely used third-generation platinum analog approved for use in the treatment of metastatic colon cancer, is reported by the Food and Drug Administration to be responsible for more than 70% rate of symptomatic neurotoxicity with any severity [11] and often leads to treatment discontinuation [12–14]. In other studies, approximately 80% of colorectal cancer patients treated with oxaliplatin alone or in combination with other chemotherapeutics experienced neurotoxicity [15–17], and impairment may be permanent. Because the number of patients being treated with a neurotoxic agent is increasing, it is essential to understand the nature of such a problematic side effect. Furthermore, testing and validating available protective strategies in preclinical and clinical settings should be the next steps in overcoming platinum-induced peripheral neurotoxicity.

Clinical features of neurotoxicity

Platinum drugs are almost always given in combination with other chemotherapy drugs and/or radiation that may be neurotoxic in their own right. Early presentation of peripheral neurotoxicity can be with numbness, tingling, or paresthesia in fingers and/or toes, a decreased distal vibratory sensitivity, and/or loss of ankle jerks [5]. Moreover, prolonged treatment may also affect proprioception, which may result in ataxic gait.

Oxaliplatin and cisplatin are the two most commonly used neurotoxic platinum agents. Platinum-induced peripheral neurotoxicity can present as two clinically distinct syndromes. The acute transient paresthesia in the distal extremities, which is only commonly seen with oxaliplatin, usually occurs within the early phase of drug administration, whereas the chronic cumulative sensory neuropathy causes more persistent clinical impairments [5]. The latter deteriorates with cumulative doses [18], followed by “coasting,” wherein symptoms worsen even months after treatment withdrawal. Furthermore, patients can develop Lhermitte's syndrome, which is a shocklike sensation of paresthesia radiating from the neck to the feet triggered by neck flexion. This phenomenon indicates the involvement of the centripetal branch of the sensory pathway within the spinal cord [19]. Neuropathy can also become irreversible. In a prospective multicenter study, Argyriou et al. [20] reported that oxaliplatin can result in an acute and chronic rate of neuropathy in 85% (169 patients of 200) and 73% (145 patients of 200) of patients, respectively.

Hearing loss or ototoxicity is another progressive and irreversible adverse effect of platinum chemotherapy [21] with a high frequency of almost 88% [22], which usually presents bilaterally and can occur during or years after treatment [23]. Nevertheless, the risk of ototoxicity may vary between cisplatin, carboplatin, and oxaliplatin treatments, and cisplatin is believed to be the most ototoxic and oxaliplatin is believed to be the least [24]. In one study, 19%–77% of patients treated with cisplatin developed bilateral sensorineural hearing loss, and 19%–42% developed permanent tinnitus [25]. Cisplatin accumulates in the cochlear tissue, forms DNA adducts, and causes inefficient and dysfunctional protein and enzyme synthesis leading to apoptosis of auditory sensory cells [26].

Diagnosis and Evaluation

The clinical diagnosis is generally not very difficult [27]. Nerve biopsies and neurophysiologic assessments are helpful for the examination of pathological and functional nerve damage (e.g., demyelinating versus axonal pathology; abnormalities in nerve conduction studies, somatosensory evoked potentials, magnetic resonance imaging, threshold tracking techniques, and quantitative sensory testing) [27]. Objective electromyography assessment of motor nerve excitability is a sensitive and specific endpoint of acute oxaliplatin-induced motor nerve hyperexcitability, which has the advantage of being widely available [28, 29]. Additionally, the threshold tracking technique is used to
assess axonal excitability [30]. This technique allows the detection of sensory axonal dysfunction before clinical symptoms [18] and can be used as a predictive marker for nerve dysfunction.

Chemotherapy-induced peripheral neurotoxicity is typically a multidisciplinary medical issue, leading to different terminology, measurement, clinical evaluation, and grading, precluding the reliability of neurological assessment. However, standardization is improving. The Functional Assessment of Cancer Therapy/Gynecologic Oncology Group-Neurotoxicity Scale, the FACT-Taxane scales, the Patient Neurotoxicity Questionnaire, European Organization for Research and Treatment of Cancer (EORTC) quality of life questionnaire [QLQ] to assess chemotherapy-induced peripheral neuropathy, and the EORTC QLQ C30 questionnaire are scoring systems that have been used for neurotoxicity assessment to quantify the impact of chemotherapy-induced neurotoxicity on patients’ quality of life [31]. Among the questionnaires, the EORTC questionnaires are widely used nowadays [32]. Among different common toxicity criteria scales that are used for peripheral neurotoxicity assessment, the one developed by the Eastern Cooperative Oncology Group and National Cancer Institute (NCI-CTC) is most widely used [19, 33–35]. Although the reliability of different assessment methods has been tested in different settings, there are vast discrepancies between patient perception and objective tools, particularly in intermediate grades [32, 36], which increase the need for a more effective and standardized method [19].

Nature of Neurotoxicity

The pathophysiology of platinum-induced peripheral neurotoxicity is not completely elucidated. Based on available data, platinum compounds may actively enter the tumor and normal cells through organic cation transporters [37], organic cation/carnitine transporters [38], and some metal transporters, such as the copper transporters [39, 40]. Platinum compounds can be excreted via platinum efflux transporters (e.g., ATP7A, ATP7B, and MRP2) [41–45] (Figure 2.1). The platinum adducts are formed...
Several studies evaluated the pharmacogenetic association of SNPs with potential functional changes [63]. Moreover, SNPs can alter the drug metabolism, cell cycle control, base excision repair, nucleotide excision repair, mismatch repair, and double-strand break repair pathways, because they may impair DNA repair pathways, including genes in single-nucleotide polymorphisms (SNPs) may play a key role in determining the induction of neurotoxicity, as well as apoptosis, because they may impair DNA repair pathways, including genes in base excision repair, nucleotide excision repair, mismatch repair, and double-strand break repair pathways [63] (Figure 2.2). Moreover, SNPs can alter the drug metabolism, cell cycle control, detoxification, or excretion pathways, which finally may lead to drug toxicities, e.g., neurotoxicity. Several studies evaluated the pharmacogenetic association of SNPs with potential functional changes.

Pharmacogenetics

Single-nucleotide polymorphisms (SNPs) may play a key role in determining the induction of neurotoxicity, as well as apoptosis, because they may impair DNA repair pathways, including genes in base excision repair, nucleotide excision repair, mismatch repair, and double-strand break repair pathways [63] (Figure 2.2). Moreover, SNPs can alter the drug metabolism, cell cycle control, detoxification, or excretion pathways, which finally may lead to drug toxicities, e.g., neurotoxicity. Several studies evaluated the pharmacogenetic association of SNPs with potential functional changes.
in the encoded protein that play a role in drug disposition, metabolism, and detoxification, DNA repair, and cancer-cell resistance and that may lead to platinum peripheral neurotoxicity [19]. However, the results are scattered and diverse with several methodological flaws, including small sample size, retrospective study design, and the implementation of a post hoc analysis of oncology-based databases of different, not preplanned sizes as well as lacking a prestudy hypothesis based on the known role of the investigated targets in the peripheral nervous system and the inappropriate outcome measures for neurological impairment [64] (Table 2.1).

There are controversial reports on the association of polymorphisms in some genes with platinum-induced neurotoxicity. These genes include ATP-binding cassette subfamily B member 1 (ABCB1) [65–68], ATP-binding cassette subfamily C member 1 or 2 (ABCC1, C2, or C2g) [69, 70], alanine-glyoxylate aminotransferase (AGXT) [69, 72, 73, 77, 94], cyclin H (CCNH) [70], catechol O-methyltransferase (COMT) [76], cytochrome P450s (CYPs; e.g., CYP2C8, CYP3A5 exons 3 and 5) [65–68], excision repair cross-complementation group 1 (ERCC1) and ERCC2 (alias XPD, xeroderma pigmentosum group D) [67, 68, 71, 74, 75, 77–87, 88], integrin β3 (ITGB3) [92], glutathione S-transferases (e.g., GSTM1 [69, 77, 78, 85, 86, 88, 89–91], GSTM3 [84, 75], and GSTT1 [88, 91]), voltage-gated sodium channel genes (SCNAs) [20, 71, 98], thiopurine S-methyltransferase (TPMT) [76], and x-ray repair cross-complementing protein 1 (XRCC1) [71, 73]. Although some data support the role of the mentioned genetic variations in the presentation and severity of platinum-induced peripheral neurotoxicity, the results are scattered and diverse (Tables 2.1, 2.2), which may form leads for future research.

ABCB1, ABCC1, ABCC2, ABCG2, and probably several other subfamily members mediate the cellular trafficking of drugs, their metabolites, and their endogenous factors, e.g., platinum efflux [99, 100]. CCNH plays an important role in the cell cycle progression, the transcriptional activity of the RNA polymerase II, and the DNA repairing process [101]. Thus, it may deregulate the repair after platinum damage to the dorsal root ganglia neurons [102]. COMT and TPMT, which encode enzymes that metabolize catecholamine-containing chemical and thiopurine drugs via methylation [103], respectively, might be associated with cisplatin-related hearing loss [104, 105].

Glutathione S-transferases (GSTs), a family of enzymes that have an important role in detoxification, have been extensively studied for the relation of SNPs with neurotoxicity induced by platinated compounds. GSTs are involved in detoxification through glutathione conjugation of electrophilic compounds (e.g., GSTM1 and GSTM3). A GSTP1 SNP (rs16953), for example, has been investigated in relation to peripheral neurotoxicity of platinum compounds in 24 studies (Table 2.1).

Among these, 9 studies reported an association of this SNP with the course and severity of peripheral neurotoxicity [68, 74, 77, 80, 85, 88–90, 93], whereas other researchers reported contradicting results in 15 studies with regard to the association of GSTP1 gene variants with neurotoxicity [67, 71, 72, 75, 78, 81, 83, 84, 86, 87, 91, 94–97]. Moreover, recent meta-analysis showed no significant associations between GSTP1 Ile105Val polymorphism and oxaliplatin-induced neuropathy in a dominant model (odds ratio [OR] = 1.08, 95% confidence interval [CI] 0.67–1.74, p = .754), a recessive model (OR = 1.67, 95% CI 0.56–4.93, p = .357), and allelic analysis (OR = 1.22, 95% CI 0.67–2.24, p = .513) [106]. This inconsistency between the findings might be explained by the difference in the cancer type, ethnicity of the population studied, and/or number of the patients enrolled in each study [19].

Other studies evaluated the association of platinum-induced peripheral neurotoxicity with different SNPs in ERCC1 [67, 68, 77, 71, 74, 78–87], ERCC2 [71, 88], and XRCC1 [71, 73], which are parts of the nucleotide excision repair (ERCC1 and ERCC2) and base excision repair (XRCC1) pathways and are required for repair of DNA lesions [107]. Although Lee et al. [73] reported the polymorphism Arg399Gln, a SNP in XRCC1 associated with less grade 2–4 sensory neuropathy in Korean patients treated with oxaliplatin-based treatment, the recent meta-analysis found it to be generally associated with poor clinical outcomes [108]. AGXT prevents accumulation of glyoxylate in the cytosol by converting it into glycolate, which is subsequently metabolized by lactate dehydrogenase into oxalate, the metabolite of...
Table 2.1. Studies on the genes with or without significant correlations with incidence and/or severity of platinum-induced peripheral neurotoxicity

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Relevance to platinum agents</th>
<th>SNP/deletion</th>
<th>With association</th>
<th>Without association</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Studies Patients</td>
<td>Studies Patients</td>
</tr>
<tr>
<td><strong>ABC</strong></td>
<td>Drug transporters (membrane efflux proteins)</td>
<td>rs2032582 1 [69] 144</td>
<td>4 [65–68] 1,591</td>
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<td></td>
<td></td>
<td>rs2074087</td>
<td></td>
<td>1 [69] 144</td>
</tr>
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<td></td>
<td></td>
<td>rs35587</td>
<td></td>
<td>1 [69] 144</td>
</tr>
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<td></td>
<td></td>
<td>rs1885301</td>
<td></td>
<td>1 [69] 144</td>
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<td></td>
<td></td>
<td>rs2273697</td>
<td></td>
<td>1 [69] 144</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs3740066</td>
<td></td>
<td>1 [69] 144</td>
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<tr>
<td></td>
<td></td>
<td>rs4148396</td>
<td></td>
<td>1 [69] 144</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs717620</td>
<td></td>
<td>1 [69] 144</td>
</tr>
<tr>
<td><strong>ABCG2</strong></td>
<td></td>
<td>rs2622604</td>
<td>1 [70] 181</td>
<td>1 [69] 144</td>
</tr>
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<td></td>
<td></td>
<td>rs3114018</td>
<td>1 [70] 181</td>
<td>1 [69] 144</td>
</tr>
<tr>
<td><strong>ACYP2</strong></td>
<td>Detoxification enzyme</td>
<td>rs34116584</td>
<td>1 [72] 135</td>
<td>3 [69, 73, 74] 518</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs44462527</td>
<td>1 [72] 135</td>
<td>3 [69, 73, 77] 570</td>
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<td></td>
<td></td>
<td>N/A del74bp</td>
<td>1 [72] 135</td>
<td>1 [69] 144</td>
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<tr>
<td><strong>BTG4</strong></td>
<td></td>
<td>rs4936453</td>
<td>2 [71] 343</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs12023000</td>
<td>2 [71] 343</td>
<td></td>
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<tr>
<td><strong>CCNH</strong></td>
<td>Cell cycle progression</td>
<td>rs2230641</td>
<td>2 [70] 206</td>
<td>1 [70] 181</td>
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<tr>
<td><strong>COMT</strong></td>
<td>Detoxification enzyme</td>
<td>rs4646316</td>
<td>1 [76] 66</td>
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<tr>
<td><strong>DLEU7</strong></td>
<td></td>
<td>rs797519</td>
<td>2 [71] 343</td>
<td></td>
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<td><strong>ERCC</strong></td>
<td>DNA repair mechanisms</td>
<td>rs11615</td>
<td>2 [74, 75] 169</td>
<td>15 [67, 68, 71, 77–88] 3,242</td>
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<tr>
<td></td>
<td></td>
<td>rs32248086</td>
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<td>1 [71] 247</td>
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<td></td>
<td></td>
<td>rs133181</td>
<td></td>
<td>1 [71] 247</td>
</tr>
<tr>
<td><strong>FARS2</strong></td>
<td></td>
<td>rs17140129</td>
<td>2 [71] 343</td>
<td></td>
</tr>
<tr>
<td><strong>FOXCl</strong></td>
<td></td>
<td>rs6924717</td>
<td>2 [71] 343</td>
<td></td>
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<tr>
<td><strong>GST</strong></td>
<td>Detoxification enzymes</td>
<td>N/A deletion</td>
<td>1 [88] 63</td>
<td>9 [69, 75, 78, 85, 86, 88–91] 1,472</td>
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<tr>
<td></td>
<td></td>
<td>N/A deletion</td>
<td>1 [84] 94</td>
<td>1 [92] 107</td>
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<tr>
<td><strong>ITG</strong></td>
<td>Cell adhesion and cell surface-mediated signaling</td>
<td>rs830884 2 [71] 343</td>
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<td></td>
<td></td>
<td>rs5918</td>
<td>1 [92] 55</td>
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<tr>
<td><strong>SCNA</strong></td>
<td>Voltage-gated sodium channels</td>
<td>rs2298771</td>
<td>1 [71] 247</td>
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<td></td>
<td></td>
<td>rs17183314</td>
<td>1 [98] 62</td>
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<tr>
<td><strong>SCN2A</strong></td>
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<td>rs2302237</td>
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<tr>
<td><strong>SCN2B</strong></td>
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<td>rs6746030</td>
<td>1 [20] 200</td>
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<tr>
<td><strong>SCN4A</strong></td>
<td></td>
<td>rs1263292</td>
<td>1 [20] 200</td>
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<tr>
<td><strong>SCN4B</strong></td>
<td></td>
<td>rs6600541</td>
<td>1 [20] 200</td>
<td></td>
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<tr>
<td><strong>TACI</strong></td>
<td>Detoxification enzyme</td>
<td>rs4380755</td>
<td>1 [76] 66</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs5008499</td>
<td>1 [76] 66</td>
<td></td>
</tr>
<tr>
<td><strong>XRCCl</strong></td>
<td>DNA repair mechanism</td>
<td>rs25487</td>
<td>1 [73] 292</td>
<td>1 [71] 247</td>
</tr>
</tbody>
</table>

Won et al. conducted the study in two settings, with 96 discovery and 247 validation samples.

Abbreviations: **ABCB1** or C1/C2/G2, ATP-binding cassette subfamily B, member 1 or C1/C2/G2; **ACYP2**, acylphosphatase 2, muscle type; **BTG4**, B-cell translocation gene 4; **AGXT**, alanine-glyoxylate aminotransferase; **CCNH**, cyclin H; **COMT**, Catechol-O-methyltransferase; **DLEU7**, deleted in lymphocytic leukemia, 7; **ERCC1**, excision repair cross-complementation group 1; **ERCC2**, alias **XPD**, Xeroderma-Pigmentosum group-D; **FARS2**, phenylalanyl-tRNA synthetase 2, mitochondrial; **FOXCl**, forkhead box C1; **GSTM1**, μ class of glutathione S-transferases; **GSTP1**, glutathione S-transferases P1/TT1; **ITGA1**, integrin, α1; **ITGB3**, integrin β3; **N/A**, not applicable; **SCNA**, voltage-gated sodium channel α-subunit; **SNP**, single nucleotide polymorphism; **TACI**, tachykinin, precursor 1; **TPMT**, thiopurine S-methyltransferase; **XRCC1**, x-ray repair cross-complementing protein 1.

Won et al. conducted the study in two settings, with 96 discovery and 247 validation samples.
### Table 2.2. Polymorphisms associated with platinum-induced peripheral neuropathy

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Cancer (patients)</th>
<th>Neurotoxic agent</th>
<th>Assessment</th>
<th>Association</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABCC1</strong></td>
<td>rs2074087</td>
<td>Colorectal (144)</td>
<td>Oxaliplatin (FOLFOX4)</td>
<td>Oxaliplatin specific scale</td>
<td>More severe PIPN for C/C or G/C vs. G/G genotypes ($p = .0170$)</td>
<td>Cecchin et al. (2013) [69]</td>
</tr>
<tr>
<td><strong>ABCC2</strong></td>
<td>rs717620</td>
<td>Colorectal (144)</td>
<td>Oxaliplatin (FOLFOX4)</td>
<td>Oxaliplatin specific scale</td>
<td>More severe PIPN for TT or C/T vs. C/C genotypes ($p = .0164$)</td>
<td>Cecchin et al. (2013) [69]</td>
</tr>
<tr>
<td></td>
<td>rs1885301</td>
<td></td>
<td></td>
<td></td>
<td>More severe PIPN for A/A or G/A vs. G/G genotypes ($p = .0072$)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs3740066</td>
<td></td>
<td></td>
<td></td>
<td>More severe PIPN for T/T or C/T vs. C/C genotypes ($p = .0231$)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs4148396</td>
<td></td>
<td></td>
<td></td>
<td>More severe PIPN for T/T or C/T vs. C/C genotypes ($p = .0048$)</td>
<td></td>
</tr>
<tr>
<td><strong>ABCG2</strong></td>
<td>rs3114018</td>
<td>Colon (181)</td>
<td>Oxaliplatin (FOLFOX/CAPOX)</td>
<td>NCI-CTC v2 or v3</td>
<td>Increased rate of severe PIPN for A/A vs. A/C vs. C/C genotypes ($p = .016$)</td>
<td>Custodio et al. (2014) [70]</td>
</tr>
<tr>
<td><strong>ACY2</strong></td>
<td>rs843748</td>
<td>Colorectal (343)</td>
<td>Oxaliplatin (FOLFOX or XELOX)</td>
<td>NCI-CTC v3</td>
<td>More severe neurotoxicity associated with G allele ($p = 1.01 \times 10^{-5}$)</td>
<td>Wo et al. (2012) [71]</td>
</tr>
<tr>
<td><strong>AGXT</strong></td>
<td>rs34116584</td>
<td>Colorectal (135)</td>
<td>Oxaliplatin (FOLFOX4)</td>
<td>NCI-CTC v1 and Oxaliplatin specific scale</td>
<td>More severe PIPN for C/T and T/T vs. C/C genotypes ($p &lt; .001$)</td>
<td>Gamelin et al. (2007) [72]</td>
</tr>
<tr>
<td></td>
<td>rs4426527</td>
<td></td>
<td></td>
<td></td>
<td>More severe PIPN for A/G and G/G vs. A/A genotypes ($p &lt; .001$)</td>
<td></td>
</tr>
<tr>
<td><strong>BTG4</strong></td>
<td>rs4936453</td>
<td>Colorectal (343)</td>
<td>Oxaliplatin (FOLFOX or XELOX)</td>
<td>NCI-CTC v3</td>
<td>More severe neurotoxicity associated with T allele ($p = 9.86 \times 10^{-5}$)</td>
<td>Wo et al. (2012) [71]</td>
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<tr>
<td><strong>CAMK2N1</strong></td>
<td>rs12023000</td>
<td>Colorectal (343)</td>
<td>Oxaliplatin (FOLFOX or XELOX)</td>
<td>NCI-CTC v3</td>
<td>More severe neurotoxicity associated with A allele ($p = 8.81 \times 10^{-5}$)</td>
<td>Wo et al. (2012) [71]</td>
</tr>
<tr>
<td><strong>CCNH</strong></td>
<td>rs2230641</td>
<td>Colon (206)</td>
<td>Oxaliplatin (FOLFOX or CAPOX)</td>
<td>NCI-CTC v3</td>
<td>Increased rate of severe PIPN for C/C vs. C/T vs. T/T ($p = .042$)</td>
<td>Custodio et al. (2014) [70]</td>
</tr>
<tr>
<td><strong>DLEU7</strong></td>
<td>rs797519</td>
<td>Colorectal (343)</td>
<td>Oxaliplatin (FOLFOX or XELOX)</td>
<td>NCI-CTC v3</td>
<td>More severe neurotoxicity associated with G allele ($p = 8.21 \times 10^{-5}$)</td>
<td>Wo et al. (2012) [71]</td>
</tr>
<tr>
<td><strong>ERCC1</strong></td>
<td>rs11615</td>
<td>Colorectal (51)</td>
<td>Oxaliplatin (FOLFOX6)</td>
<td>NCI-CTC v3</td>
<td>Higher incidence of PIPN (grade 1) for C/T and T/T vs. C/C genotypes ($p = .016$)</td>
<td>Inada et al. (2010) [74]</td>
</tr>
<tr>
<td></td>
<td>rs3212986</td>
<td>Ovarian (118)</td>
<td>Cisplatin/carboplatin (+ paclitaxel/docetaxel)</td>
<td>NCI-CTC v2</td>
<td>Higher incidence of PIPN (grades 3–4) for C/C vs. C/A or A/A genotypes ($p = .019$)</td>
<td>Kim et al. (2009) [75]</td>
</tr>
<tr>
<td><strong>FARS2</strong></td>
<td>rs17140129</td>
<td>Colorectal (343)</td>
<td>Oxaliplatin (FOLFOX or XELOX)</td>
<td>NCI-CTC v3</td>
<td>More severe neurotoxicity associated with A allele ($p = 3.23 \times 10^{-5}$)</td>
<td>Wo et al. (2012) [71]</td>
</tr>
<tr>
<td></td>
<td>rs6924717</td>
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<td></td>
<td></td>
<td>More severe neurotoxicity associated with C allele ($p = 3.23 \times 10^{-5}$)</td>
<td>Wo et al. (2012) [71]</td>
</tr>
<tr>
<td><strong>FOX1</strong></td>
<td>rs2338</td>
<td>Colorectal (343)</td>
<td>Oxaliplatin (FOLFOX or XELOX)</td>
<td>NCI-CTC v3</td>
<td>More severe neurotoxicity associated with G allele ($p = 4.63 \times 10^{-5}$)</td>
<td>Wo et al. (2012) [71]</td>
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<td><strong>GSTM1</strong></td>
<td>Deletion</td>
<td>Colorectal (63)</td>
<td>Oxaliplatin (mFOLFOX-6)</td>
<td>NCI-CTC v3</td>
<td>More severe PIPN (grades 2–3) for A/G or G/G vs. A/A genotypes ($p = .03$)</td>
<td>Kumamoto et al. (2013) [88]</td>
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<tr>
<td><strong>GSTM3</strong></td>
<td>rs1799735</td>
<td>Ovarian (104)</td>
<td>Cisplatin</td>
<td>NCI-CTC$^b$</td>
<td>Lower incidence of PIPN for AGG/AGG genotype ($p = .055$)</td>
<td>Khrunin et al. (2010) [84]</td>
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</table>

(continued)
Table 2.2. Continued

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<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Cancer (patients)</th>
<th>Neurotoxic agent</th>
<th>Assessment</th>
<th>Association</th>
<th>Reference</th>
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<tr>
<td>GSTP1</td>
<td>rs16953</td>
<td>Colorectal (52)</td>
<td>Oxaliplatin</td>
<td>NCI-CTC v3</td>
<td>Higher incidence of PIPN (grades 2–3) for A/G or G/G vs. A/A genotypes (p = .03)</td>
<td>Hong et al. (2011) [77]</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Oxaliplatin (FOLFOX4)</td>
<td>NCI-CTCb</td>
<td>Higher incidence of PIPN: for G/G and A/G vs. A/A genotypes (p &lt; .01)</td>
<td>Chen et al. (2010) [80]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Colorectal (51)</td>
<td>Oxaliplatin (FOLFOX6)</td>
<td>NCI-CTC v3</td>
<td>Higher incidence of PIPN (grade 1) for A/A vs. A/G and G/G genotypes (p = .032)</td>
<td>Inada et al. (2010) [74]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Oxaliplatin (FOLFOX4 or IROX)</td>
<td>NCI-CTC v2</td>
<td>More severe PIPN (grades 3–4) for T/T genotype (p = .003)</td>
<td>McLeod et al. (2010) [68]</td>
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<td></td>
<td></td>
<td>Colorectal (166)</td>
<td>Oxaliplatin (FOLFOX4)</td>
<td>Oxaliplatin specific scale</td>
<td>More severe PIPN for G/G &gt; A/G &gt; A/A genotypes (p &lt; .001)</td>
<td>Ruzzo et al. (2007) [85]</td>
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<td></td>
<td>Colorectal (59)</td>
<td>Oxaliplatin</td>
<td>Oxaliplatin specific scale</td>
<td>Higher PIPN incidence (grade 3) for A/A vs. A/G and G/G genotypes (p = .02)</td>
<td>Lecomte et al. (2006) [89]</td>
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<td></td>
<td></td>
<td>Colorectal (66)</td>
<td>Oxaliplatin (mFOLFOX-6)</td>
<td>NCI-CTC v3</td>
<td>More severe PIPN (grades 2–3) for A/G or G/G vs. A/A genotypes (p = .05)c</td>
<td>Kumamoto et al. (2013) [88]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gastric (85)</td>
<td>Oxaliplatin (FOLFOX6)</td>
<td>NCI-CTC v2</td>
<td>More severe PIPN for A/A vs. A/G and G/G genotypes (p = .005)</td>
<td>Li et al. (2010) [93]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Testicular (238)</td>
<td>Cisplatin (+ bleomycin, etoposide or vinblastine)</td>
<td>SCIN</td>
<td>More severe PIPN for A/A genotype (p = .012) or A/G vs. G/G genotypes (p = .003)</td>
<td>Oldenburg et al. (2007) [90]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ITGA1</th>
<th>rs830884</th>
<th>Colorectal (343)a</th>
<th>Oxaliplatin (FOLFOX or XELOX)</th>
<th>NCI-CTC v3</th>
<th>More severe neuropathy associated with T allele (p = 1.74 × 10⁻³)</th>
<th>Wo et al. (2012) [71]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITGB3</td>
<td>rs5918</td>
<td>Colorectal (55)</td>
<td>Oxaliplatin</td>
<td>TNS</td>
<td>More severe PIPN for T/T genotype vs. C/T and C/C genotypes (p = .044)</td>
<td>Antonacopoulou et al. (2010) [92]</td>
</tr>
<tr>
<td>SCNA</td>
<td>rs2302237</td>
<td>Colorectal (200)</td>
<td>Oxaliplatin (FOLFOX4)</td>
<td>NCI-CTC v3 and TNS</td>
<td>More severe (p = .0029) and higher incidence of acute (p = .019) and chronic (p = .037) PIPN for C/T vs. C/C and T/T genotypes</td>
<td>Argyriou et al. (2013) [20]</td>
</tr>
<tr>
<td></td>
<td>rs1263292</td>
<td></td>
<td></td>
<td></td>
<td>Higher incidence of PIPN for C/T vs. C/C and T/T genotypes (p = .023)</td>
<td></td>
</tr>
<tr>
<td>TACI</td>
<td>rs10486003</td>
<td>Colon (292)</td>
<td>Oxaliplatin</td>
<td>NCI-CTC v3</td>
<td>More severe neuropathy associated with C allele (p = 4.84 × 10⁻³)</td>
<td>Lee et al. (2013) [73]</td>
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<tr>
<td>XRCC1</td>
<td>rs23885</td>
<td>Colon (292)</td>
<td>Oxaliplatin</td>
<td>NCI-CTC v3</td>
<td>Less severe (p = .050)c and latter-onset (p = .041) PIPN for A/G and A/A vs. G/G genotype</td>
<td></td>
</tr>
</tbody>
</table>

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*This study has been conducted in two settings, with 96 discovery and 247 validation samples. The p values mentioned are for the combined groups, which were also significant for each SNP in each group.

*Version of the scoring scale was not reported.

*Trends toward significance.

Abbreviations: ABCG2, ATP-binding cassette subfamily G, member 2; AGXT, alanine-glyoxylate aminotransferase; Btg4, B-cell translocation gene 4; CAMK2N1, calcium/calmodulin-dependent protein kinase II inhibitor 1; CAPOX, capecitabine and oxaliplatin; CCNH, cyclin H; DLEU7, deleted in lymphocytic leukemia, 7; ERCC1, excision repair cross-complementing group 1; FARS2, phosphorylalanyl-tRNA synthetase 2, mitochondrial; FOLFOX, folinic acid (leucovorin), fluorouracil and oxaliplatin; FOXC1, forkhead box C1; GSTM1, glutathione S-transferases; GSTP1, glutathione S-transferases P1; IROX, irinotecan plus oxaliplatin; ITA1, integrin α1; ITGB3, integrin β3; mFOLFOX, modified FOLFOX; NCI-CTC, National Cancer Institute–Common Toxicity Criteria; PIPN, platinum-induced peripheral neurotoxicity; SCIN, scale for chemotherapy-induced long-term neurotoxicity; SCNA, voltage-gated sodium channel α-subunit; SNP, single-nucleotide polymorphism; TAC1, tachykinin, precursor 1; TNS, total neuropathy score; v, version; XELOX, oxaliplatin, capecitabine; XRCC2, x-ray repair cross-complementing protein 1.
oxaliplatin [109]. Pharmacogenetic analyses evaluated also cytochrome P450s [65–68], which are major enzymes of drug metabolism and bioactivation (e.g., CYP2C8 and CYP3A5), and ITGB3 [92], which belongs to the large family of integrins, known to participate in cell adhesion and cell surface-mediated signaling.

A recent study has provided evidence that SNPs in voltage-gated sodium channel genes (SCNAs; e.g., SCN4A-rs2302237 and SCN10A-rs1263292) can play a causal role in oxaliplatin-based peripheral neurotoxicity [20, 57] (Table 2.1). A polymorphism in SCN1A (rs3812718) was also reported to be associated with decreased neurotoxicity [85]. However, these results still need to be validated by appropriate larger and prospective studies. Won et al. [71], in a genome-wide pharmacogenomic approach, identified nine novel polymorphisms associated with and predictors of severe oxaliplatin-induced peripheral neurotoxicity, including rs10486003 (tachykinin, precursor 1 [TAC1]), rs2338 (forkhead box C1 [FOXC1]), rs830884 (integrin α1 [ITGA1]), rs843748 (acylphosphatase 2, muscle type [ACYP2]), rs4936453 (B-cell translocation gene 4 [BTG4]), rs17140129 and rs6924717 (phenylalanyl-tRNA synthetase 2 [FARS2]), rs12023000 (calcium/calmodulin-dependent protein kinase II inhibitor 1 [CAMK2N1]), and rs797519 (deleted in lymphocytic leukemia, 7 [DLEU7]) [71]. These genes may account for the mechanism of neurotoxicity prevention by calcium-magnesium infusions or may be associated with the important oxalate and glyoxylate outcome pathway [72]. However, none of the SNPs in the discovery samples (96 patients with colon cancer) surpassed genome-wide significance, and these SNPs were not significant in their validation set (247 patients with colorectal cancer; \( p = .05–.19 \)) [71]. However, the authors noted that this limitation might be overcome by increasing the sample size in a prospective analysis.

Some evidence demonstrated that mitogen-activated protein kinase pathways, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (Sapk), and p38, might also have a causal effect on chemotherapy-induced peripheral neuropathies [110]. Normally, there is a balance between ERK1/2 and p38 activation, which regulates neuronal apoptosis, and JNK/Sapk, which preserves neuronal degeneration. This balance is also altered by platinum derivatives [109].

**Neuroprotection**

**Neuroprotective Agents, Mechanisms and Controversies**

Several model systems have been used to study the nature of overall neurotoxicity and the effect of potential neuroprotective drugs. These include overall neurotoxicity signs in the animal, specific models including the DRG of rats [111–113], the structure of the cerebral ganglia of snails [114–116], in vitro models such as neurite extension [117–121], and evaluation of biomarkers of neurotoxicity such as cyclin B [122]. These models have been very useful to select proper potential neuroprotective drugs to be evaluated in the clinic. Unfortunately, preventive and therapeutic treatment options are not sufficient so far to bypass neurotoxicity [9, 19, 123]. However, a few drugs can, to some extent, protect against platinum-induced peripheral neurotoxicity (Table 2.3). Neuroprotective drugs include the following.

**Detoxicants**

Sodium thiosulfate (STS) is a reactive thiol agent used clinically as an antidote to cyanide or nitroprusside poisoning, and at high molar excess, it binds to and inactivates the electrophilic platinum compound. Its use includes otoprotection [169–173] (discussed separately under “Neuro- Versus Chemoprotection”).
Table 2.3. Randomized controlled trials on neuroprotective agents for the prevention of platinum-induced peripheral neurotoxicity

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mechanism of action</th>
<th>Total patients (treated vs. control)/tumor type</th>
<th>Neurotoxic agent(s)</th>
<th>Neuroprotection</th>
<th>Study design/overall risk of biasa</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAC</td>
<td>A nutritional supplement thought to increase whole blood concentrations of glutathione; it also activates ERKs and p38 MAPK, to mediate the neuroprotective effect of NAC</td>
<td>14 (5.9)/colorectal cancer</td>
<td>Oxaliplatin</td>
<td>Decrease in incidence and severity of grades 2–4 neurotoxicity assessed by NCI-CTC ($p &lt; .05$); no significant changes in incidence and mean SNAP amplitude</td>
<td>Prospective, randomized, placebo-controlled/intermediate (dissimilarity of groups, small-sized trial, randomization, blinding, concealment allocation, outcome data not described, etc.)</td>
<td>Lin et al. 2006 [124]</td>
</tr>
<tr>
<td>α-Lipoic acid</td>
<td>A physiologic antioxidant with neuroprotective activity</td>
<td>243 (122:121); 29% completed the trial (34: 36)/gastrointestinal cancers (176), lung cancer (27), genitourinary cancers (14), other (15)</td>
<td>Cisplatin or oxaliplatin</td>
<td>No significant decrease in incidence and severity of neurotoxicity assessed by NCI-CTC, FACT/GOG-Ntx, and BPI score</td>
<td>Prospective, randomized, double-blind, placebo-controlled trialb</td>
<td>Guo et al. 2014 [125]</td>
</tr>
<tr>
<td>Amifostine</td>
<td>Detoxicant: an organic thiophosphate cystamine analog with cytoprotective activity</td>
<td>92 (46:46)/advanced or relapsed colorectal or gastric cancer with variable metastatic disease to liver, lung, and other sites</td>
<td>Oxaliplatin (FOLFOX4)</td>
<td>Decrease in incidence of grades 1–4 neurotoxicity assessed by NCI-CTC ($p &lt; .007$); changes in severity and neurophysiologic assessment NR</td>
<td>Prospective, randomized, placebo controlled trial/low (blinding and concealment allocation not described)</td>
<td>Lu et al. 2008 [126]</td>
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<td>90 (45:45)/advanced ovarian carcinoma</td>
<td>Carboplatin, paclitaxel</td>
<td>Decrease in incidence of neurotoxicity grades 2–3 assessed by NCI-CTC ($p &lt; .05$); changes in severity and neurophysiologic assessment NR</td>
<td>Randomized phase II study/high (nonplacebo trial, observers unblinded unclear participant blinding, and incomplete data reporting, randomization and concealment allocation not described)</td>
<td>De Vos et al. 2005 [127]</td>
</tr>
<tr>
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<td>72 (37:34)/ovarian cancer</td>
<td>Carboplatin, paclitaxel</td>
<td>A significant protective effect observed for 2-PD, TRA, VPT, VDT, and NCI-CTC neuropathy scores</td>
<td>Randomized double-blind placebo-controlled trial/low (unable to separate the effects of carboplatin from those of paclitaxel, similarity of groups unclear, etc.)</td>
<td>Hilpert et al. 2005 [128]</td>
</tr>
<tr>
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<td>38 (19:19)/NSCLC</td>
<td>Carboplatin, paclitaxel</td>
<td>Decrease in incidence of grades 1–2 neurotoxicity assessed by NCI-CTC ($p = .018$); no significant decline in mean SNAP amplitudes</td>
<td>Prospective, randomized/intermediate (small sample size, unblinded participants, no placebo, incomplete outcome data, etc.)</td>
<td>Kanat et al. 2003 [129]</td>
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<tr>
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<td></td>
<td>60 (30:30)/unresectable stage III NSCLC</td>
<td>Carboplatin, paclitaxel</td>
<td>No significant changes in incidence or neurophysiologic</td>
<td>Randomized double-blind trial/low (only two doses of carboplatin, small sample size, etc.)</td>
<td>Leong et al. 2003 [130]</td>
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(continued)
### Table 2.3. (Continued)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mechanism of action</th>
<th>Total patients (treated vs. control)/tumor type</th>
<th>Neurotoxic agent(s)</th>
<th>Neuroprotection</th>
<th>Study design/overall risk of bias</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>187 (93:94)/advanced ovarian cancer</td>
<td>Carboplatin, paclitaxel</td>
<td>Decrease in severity of grades 3–4 neurotoxicity; assessed by NCI-CTC ($p = .021$); changes in incidence and severity NR</td>
<td>Phase III prospective multicenter randomized trial/low (not placebo controlled, unclear blinding, incomplete outcome data etc.)</td>
<td>Lorusso et al. 2003 [131]</td>
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<tr>
<td>20 (10:10)/cervical cancer</td>
<td>Cisplatin</td>
<td>No significant changes in incidence and severity of neuropathy</td>
<td>An open, single-blinded pilot study/high (open, single-blinded, no allocation concealment, small trial, etc.)</td>
<td>Gallardo et al. 1999 [132]</td>
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</tr>
<tr>
<td>73 (36:37)/advanced head and neck cancer</td>
<td>Cisplatin</td>
<td>Decrease in incidence of subclinical neurotoxicity assessed by VPT ($p = .03$); changes reported in severity NR</td>
<td>Prospective, randomized, placebo-controlled/intermediate (unclear blinding, randomization and allocation concealment, etc.)</td>
<td>Planting et al. 1999 [133]</td>
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<tr>
<td>242 (122:120)/ovarian cancer</td>
<td>Cisplatin</td>
<td>Decrease in severity of grades 1–3 neurotoxicity ($p = .029$); assessed by NCI-CTC; incidence and neurophysiologic assessment NR</td>
<td>Prospective, randomized/low (not placebo-controlled, etc.)</td>
<td>Kemp et al. 1996 [134]</td>
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<tr>
<td>BNP7787</td>
<td>Detoxicant: neuroprotective</td>
<td>151 (76:75)/advanced NSCLC</td>
<td>Cisplatin, docetaxel</td>
<td>Unable to separate the effects of cisplatin from those of docetaxel</td>
<td>Phase II randomized study (unblinded and not placebo-controlled)</td>
<td>Miller et al. 2008 [135]</td>
</tr>
<tr>
<td>Calcium-magnesium</td>
<td>Chelate with oxaliplatin metabolite and protect the voltage-gated sodium channels from alteration</td>
<td>20 (10:10)/colorectal cancer</td>
<td>Oxaliplatin (XELOX or mFOLFOX6)</td>
<td>No significant decrease in severity of neurotoxicity based on motor nerve excitability assessed by EMG</td>
<td>Randomized, double-blinded, placebo-controlled, crossover study</td>
<td>Han et al. 2013 [136]</td>
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<td>353 (118:116:119) divided into three groups of CaMg/CaMg; CaMg/placebo; placebo/placebo before and after chemotherapy/colon cancer</td>
<td>Oxaliplatin (FOLFOX4 or 6)</td>
<td>No significant changes in severity of grades 2–4 assessed by NCI-CTC; no significant changes in EORTC CIPN-20 sensory, motor, or autonomic scales; changes in incidence and neurophysiologic assessment NR</td>
<td>Phase III randomized, placebo-controlled, double-blind study/low</td>
<td>Loprinzi et al. 2014 [137]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>102 (50:52)/colorectal cancer</td>
<td>Oxaliplatin</td>
<td>Lower incidence of grades 2–4 neurotoxicity; assessed by NCI-CTC ($p = .018$); changes in severity</td>
<td>Prospective, randomized, placebo-controlled, double-blind/low (insufficient sample size)</td>
<td>Grothey et al. 2011 [138]</td>
</tr>
<tr>
<td>Agent</td>
<td>Mechanism of action</td>
<td>Total patients (treated vs. control)/tumor type</td>
<td>Neurotoxic agent(s)</td>
<td>Neuroprotection</td>
<td>Study design/overall risk of bias</td>
<td>Reference</td>
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<tr>
<td>Carbamazepin</td>
<td>A sodium channel blocker that exerts its effect by inhibiting hyperexcitability of the channel on oxaliplatin</td>
<td>36 (19:17)/advanced colorectal cancer</td>
<td>Oxaliplatin (FOLFOX)</td>
<td>No significant changes in incidence and severity of neurotoxicity assessed by Levi’s scale</td>
<td>Randomized, controlled multicenter phase II study/low (small sample size)</td>
<td>von Delius et al. 2007 [142]</td>
</tr>
<tr>
<td>Diethyldithio-carbamate</td>
<td>Chelating agent and antioxidant: prevents the degradation of extracellular matrix, as an initial step in cancer metastasis and angiogenesis</td>
<td>195 (96:99)/ovarian, SCLC, and NSCLC</td>
<td>Cisplatin</td>
<td>Adverse effects were reported in all study participants and lower levels of cisplatin administration; changes in severity and neurophysiologic assessment NR</td>
<td>Prospective, randomized, placebo controlled multicenter trial/low (insufficient sample size considering the variety of center types, etc.)</td>
<td>Gandara et al. 1995 [143]</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Neurotrophic: induces NGF synthesis</td>
<td>86 (42:44)/metastatic colorectal cancer</td>
<td>Oxaliplatin</td>
<td>Lower incidence of grades 1−2 neurotoxicity; assessed by NCI-CTC (p = .04); severity assessment NR; no significant changes in neurophysiologic assessment</td>
<td>Randomized pilot study/high (neither blinded nor placebo-controlled)</td>
<td>Wang et al. 2007 [144]</td>
</tr>
<tr>
<td>Glutathione</td>
<td>Antioxidant: a physiologic free radical scavenger, chelator for heavy metals that decreases the initial accumulation of platinum adducts in the neurons,</td>
<td>185 (94:91)/ovarian, fallopian tube, and primary peritoneal cancers (86), lung cancer (53), other types (22)</td>
<td>Carboplatin, paclitaxel</td>
<td>No significant changes in incidence and severity of neuropathy grades 1−4 assessed by EORTC QLQ-CIPN20, and NCI-CTC reported; changes in</td>
<td>Phase III randomized, double-blind placebo-controlled study/low</td>
<td>Leal et al. 2014 [145]</td>
</tr>
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</table>

Table 2.3. (Continued)
<table>
<thead>
<tr>
<th>Agent</th>
<th>Mechanism of action</th>
<th>Total patients (treated vs. control)/tumor type</th>
<th>Neurotoxic agent(s)</th>
<th>Neuroprotection</th>
<th>Study design/overall risk of bias</th>
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<tbody>
<tr>
<td>and modulator of MAPKs,</td>
<td>and modulator of MAPKs, including JNK/Sapk ERKs and p38 pathways with which</td>
<td></td>
<td></td>
<td>neurophysiologic assessment NR</td>
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<td>including JNK/Sapk ERKs and</td>
<td>apoptosis is inhibited</td>
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<td>p38 pathways with which</td>
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<td>apoptosis is inhibited</td>
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<tr>
<td>27 (14:13)/colorectal cancer</td>
<td>Oxaliplatin (FOLFOX4)</td>
<td>Lower incidence of grades 1 and 3 neurotoxicity</td>
<td>assessed by NCI-CTC ($p = .0037$); changes in severity and neurophysiologic assessment NR</td>
<td>Prospective, randomized, placebo-controlled/intermediate (small sample size, unclear randomization and blinding, etc.)</td>
<td>Milla et al. 2009 [146]</td>
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</tr>
<tr>
<td>52 (26:26)/colorectal cancer</td>
<td>Oxaliplatin</td>
<td>Decrease in incidence and severity of grades 1–4 neurotoxicity; assessed by NCI-CTC, and sensory nerve conduction studies (sural, median, or ulnar); severity assessment NR; no significant changes in neurophysiologic assessment</td>
<td>Prospective, randomized placebo-controlled/low (incomplete outcome data, etc.)</td>
<td>Cascinu et al. 2002 [147]</td>
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<tr>
<td>20 (11:9)/NSCLC and head</td>
<td>Cisplatin</td>
<td>No significant decrease in incidence of neurotoxicity assessed by WHO neurotoxicity measure and motor nerve conduction studies</td>
<td>Prospectively randomized placebo-controlled pilot trial/intermediate (insufficient sample size, unclear randomization, and blinding)</td>
<td>Schmidinger et al. 2000 [148]</td>
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<td>and neck cancer</td>
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<td>151 (74:77)/ovarian cancer</td>
<td>Cisplatin</td>
<td>Significantly less tingling assessed by NCI/WHO; HAD and Rotterdam scales (functional scales); changes in severity and neurophysiologic assessment NR</td>
<td>Prospective, randomized, placebo-controlled/low (blinding and outcome data not clearly described, etc.)</td>
<td>Smyth et al. 1997 [149]</td>
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<tr>
<td>50 (25:25)/ovarian cancer</td>
<td>Cisplatin</td>
<td>Decrease in incidence of neurotoxicity assessed by NCI/WHO criteria and sensory nerve conduction studies (sural, median, or ulnar; $p &lt; .01$); changes in severity NR</td>
<td>Prospective, randomized, placebo-controlled/low (relatively small trial, long-term follow-up unclear, incomplete outcome data, etc.)</td>
<td>Cascinu et al. 1995 [150]</td>
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<tr>
<td>33 (16:17)/relapsing ovarian cancer</td>
<td>Cisplatin</td>
<td>No significant changes in incidence and severity of neuropathy assessed by SNAP and NCI/WHO</td>
<td>Prospective, randomized, placebo-controlled study/low (lack of information about randomization and blinding and small sample)</td>
<td>Colombo et al. 1995 [151]</td>
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<table>
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<tr>
<th>Agent</th>
<th>Mechanism of action</th>
<th>Total patients (treated vs. control)/tumor type</th>
<th>Neurotoxic agent(s)</th>
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<th>Study design/overall risk of bias</th>
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<td>Decrease in SNAP amplitude (sural, median, or ulnar; ( p = .043 )) and VPT; changes in severity of neurotoxicity not significant; changes in incidence NR</td>
<td>Prospective, randomized, placebo-controlled/intermediate (unblinded, relatively small trial, unclear randomization and allocation concealment, reporting bias, etc.)</td>
<td>Bogliun et al. 1992 [152]</td>
</tr>
<tr>
<td>Goshajinkigan (Kampo medicine; composed of 10 natural ingredients)</td>
<td>Acts via release of dynorphin, nitric-oxide production, and reducing transmitter proteins and sensory receptors associated with C-fiber activation</td>
<td>89 (44:45)/colorectal cancer</td>
<td>Oxaliplatin (FOLFOX4 or mFOLFOX6)</td>
<td>Suggestive of a decrease in incidence of grade 2–3 neurotoxicity assessed by NCI-CTC and FACT/GOG-Ntx, but insignificant; changes in severity and neurophysiologic assessment NR</td>
<td>Phase II, multicenter, randomized, double-blind, placebo-controlled trial</td>
<td>Kono et al. 2013 [153]</td>
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<td>Lower incidence of grades 3 neurotoxicity; assessed by DEB-NTC ( p &lt; .01 ); unblinded control; Severity and neurophysiologic assessment NR</td>
<td>Prospective randomized controlled study/low (relatively small sample size with unblinded control group, etc.)</td>
<td>Nishioka et al. 2011 [154]</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>Calcium channel antagonist that might attenuate chelating effect by increasing calcium serum levels</td>
<td>50 (24:26)/ovarian cancer</td>
<td>Cisplatin</td>
<td>The trial was terminated because of side effects; the available data suggested that nimodipine exacerbated (not prevented) neurotoxicity; incidence, severity and neurophysiologic assessment NR</td>
<td>Randomized placebo-controlled study/high (small trial with unreliable measures and inadequate follow-up period)</td>
<td>Cassidy et al. 1998 [155]</td>
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<td>No significant changes in incidence and severity of neurotoxicity; VPT increased in both groups during the study (worse outcome)</td>
<td>Prospective, randomized, placebo-controlled/low (unclear randomization and allocation concealment, etc.)</td>
<td>Roberts et al. 1997 [156]</td>
</tr>
<tr>
<td>Org 2766</td>
<td>Neurotrophic: an adrenocorticolotropic hormone analog with neurotrophic effects</td>
<td>196 (129:67)/epithelial ovarian cancer</td>
<td>Cisplatin</td>
<td>Neurophysiologic assessment not suggestive of neuroprotection; changes</td>
<td>Prospective, randomized, placebo-controlled/low (insufficient sample size, unclear randomization and allocation concealment, etc.)</td>
<td>van Gerven et al. 1994 [157]</td>
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### Table 2.3. (Continued)

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<tr>
<th>Agent</th>
<th>Mechanism of action</th>
<th>Total patients (treated vs. control)/tumor type</th>
<th>Neurotoxic agent(s)</th>
<th>Neuroprotection</th>
<th>Study design/overall risk of bias*</th>
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</tr>
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<tr>
<td>Cisplatin VPT</td>
<td>Neurotoxic agent(s) Neuroprotection</td>
<td>20 (7:11)/epithelial ovarian cancer</td>
<td>Cisplatin</td>
<td>VPT increased significantly less in active arm than placebo; changes in incidence and severity NR</td>
<td>Prospective, randomized, placebo-controlled/intermediate (insufficient sample size, unclear randomization, substantial dropout, incomplete outcome data, etc.)</td>
<td>Hovestadt et al. 1992 [158]</td>
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<tr>
<td>Cisplatin VPT</td>
<td>VPT increased approximately twofold, significantly less than the nearly eightfold increase in the placebo group; changes in incidence and severity NR</td>
<td>55 (33:22)/epithelial ovarian cancer</td>
<td>Cisplatin</td>
<td>VPT increased significantly less in active arm than placebo; changes in incidence and severity NR</td>
<td>Prospective, randomized, placebo-controlled/low (relatively small trial, etc.)</td>
<td>van der Hoop et al. 1990 [159]</td>
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<td>Oxcarbazepine</td>
<td>Sodium channel inhibitor: protects the alteration of voltage-gated sodium channels by oxalate</td>
<td>40 (20:20)/colon cancer</td>
<td>Cisplatin</td>
<td>Significant decrease in incidence and severity of neurotoxicity assessed by NSS, TNS, SNAP, and peroneal motor responses</td>
<td>Prospective, randomized, open label with blind assessment/intermediate (relatively small trial with an unblinded control arm, etc.)</td>
<td>Argyriou et al. 2006 [160]</td>
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<td>Retinoic acid (all-trans-retinoic acid)</td>
<td>Stimulator of NGF and the expression of its receptor, activator of retinoic acid receptors with neuroprotective profile; it is also a prodifferentiative agent able to activate both JNK/Sapk and ERK1/2</td>
<td>92 (45:47)/advanced NSCLC</td>
<td>Cisplatin, paclitaxel</td>
<td>Decrease in incidence and severity of grade 2–4 neuropathy assessed by motor and sensory response amplitudes and NCI-CTC</td>
<td>Prospective, randomized, placebo-controlled/low (short period of evaluation, etc.)</td>
<td>Arrieta et al. 2005 [161]</td>
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<tr>
<td>rhuLIF</td>
<td>Neurotrophic: a IL-6 class cytokine that upregulates NGF synthesis, which affects nerve regeneration by inhibiting differentiation</td>
<td>117 (75:42)/different solid tumors</td>
<td>Carboplatin, paclitaxel</td>
<td>Worse neuropathy endpoints in the active therapy arm for changes in velocity in the median nerve; no significant changes in CPNE score and VPT; changes in incidence of neuropathy NR</td>
<td>Randomized, double-blinded, placebo-controlled phase II trial/low</td>
<td>Davis et al. 2005 [162]</td>
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<tr>
<td>Venlafaxine</td>
<td>Serotonin-norepinephrine reuptake inhibitor: modulates the oxidative stress in the nervous system and may block sodium channels</td>
<td>48 (24:24)/different solid tumors</td>
<td>Carboplatin/paclitaxel</td>
<td>Decrease in NPSI (pins and needles; p &lt; .001); no significant decrease in NPSI pain triggered by cold; changes in incidence and neurophysiologic assessment NR</td>
<td>Randomized, double-blind, placebo-controlled phase III trial/intermediate (small sample size, unclear allocation concealment, etc.)</td>
<td>Durand et al. 2012 [163]</td>
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<tr>
<td>Vitamin E</td>
<td>Antioxidant: protects biologic membranes by inhibiting peroxidation of polyunsaturated fatty acids, and a protector against platinum accumulation in the DRG</td>
<td>189 (96:93)/colorectal, breast, lung, and other cancers</td>
<td>Cisplatin (8), carboplatin (2), oxaliplatin (50), taxanes (109), or combinations of each</td>
<td>Inclusion of a large number of participants receiving taxanes was confounding; changes in neurophysiologic assessment NR</td>
<td>Randomized, double-blind, placebo-controlled phase III trial/low (different types of cancers with no comparable distribution and insufficient sample size for platinum groups, etc.)</td>
<td>Kottschade et al. 2011 [164]</td>
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<td>108 (54:54) after dropout (17:24)/different solid tumors</td>
<td>Cisplatin</td>
<td>Significant decrease in incidence and severity of neurotoxicity assessed by TNS and SNAP (sural and median)</td>
<td>Randomized, placebo-controlled trial/high (substantial dropout rate, excluding intention-to-treat analyses, etc.)</td>
<td>Pace et al. 2010 [165]</td>
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<td>30 (14:16)/solid tumors</td>
<td>Cisplatin, paclitaxel</td>
<td>Significant decrease in incidence and severity of neurotoxicity assessed by NSS/NDS; reduced median SNAP amplitudes in control group</td>
<td>Prospective, randomized, open label with blind assessment/high (relatively small trial and lack of placebo group and reliable measures; excluding intention-to-treat analyses, etc.)</td>
<td>Argyriou et al. 2006 [166]</td>
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<td>27 (13:14)/lung, ovarian, rhino-pharyngeal, gastric, testicular, esophagus, ethmoid, and tongue cancers</td>
<td>Cisplatin</td>
<td>Significant decrease in incidence and severity of neurotoxicity; reduced median SNAP amplitudes in control group</td>
<td>Prospective, randomized, placebo-controlled/high (small size and excluding intention-to-treat analyses; control participants were untreated)</td>
<td>Pace et al. 2003 [167]</td>
</tr>
<tr>
<td>Xaliproden</td>
<td>A synthetic neuromodulant that activates MAPK pathways, which minimizes neuritic damage</td>
<td>649 (325:324)/metastatic colorectal cancer</td>
<td>Oxaliplatin (FOLFOX4)</td>
<td>Efficient in reducing grade 3–4 neurotoxicity evaluated by sensory action potential (17% vs. 11% with placebo); no significant difference in overall incidence (73% of both arms)</td>
<td>Randomized, double-blind, placebo-controlled phase III trial (unpublished data)</td>
<td>Cassidy et al. 2006 [168]</td>
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*Strength of evidence is according to the 2014 American Society of Clinical Oncology Clinical Practice Guideline [123] and 2014 Cochrane systematic review [9].

*Not assessed by American Society of Clinical Oncology or Cochrane studies.

Abbreviations: 2-PD, two-point discrimination; CPNE, composite peripheral nerve electrophysiology; DEB-NTS, Debiopharm Neurotoxicity Scale; DRG, dorsal root ganglion; EMG, electromyography; EORTC QLQ-CIPN20, European Organization for Research and Treatment of Cancer quality of life questionnaire to assess chemotherapy-induced peripheral neuropathy; ERK, extracellular signal-regulated kinase; FACT/GOG-Ntx, Functional Assessment of Cancer Therapy/Gynecologic Oncology Group-Neurotoxicity Scale; FOLFOX, folinic acid (leucovorin), fluorouracil, and oxaliplatin; HAD, hospital anxiety and depression; IL, interleukin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; mFOLFOX, modified FOLFOX; NAC, N-acetylcysteine; NCI-CTC, National Cancer Institute–Common Toxicity Criteria; NCS, nerve conduction studies; NDS, neurological disability score; NGF, nerve growth factor; NIS, neuropathy impairment scale; NPSI, Neuropathic Pain Symptom Inventory; NR, not reported; NSCLC, non-small cell lung cancer; OSS, oxaliplatin-specific scale; rhuLIF, recombinant human leukemia inhibitory factor; Sapk, stress-activated protein kinase; SCLC, small cell lung cancer; SNAP, sural, superficial peroneal, and ulnar sensory nerve action potential; TNS, total neuropathy score; TRA, tendon reflex activity; VDT, vibration disappearance threshold; VPT, vibration perception threshold; WHO, World Health Organization; XELOX, oxaliplatin and capecitabine.
Amifostine is an organic thiophosphate also regarded as a cytoprotective and detoxicant agent [117]. There are successful in vitro results supporting amifostine neuroprotection against cisplatin [120], as well as against oxaliplatin [122].

Some clinical data, with different levels of reliability (Table 2.3), indicated that amifostine exerts some protection against peripheral neurotoxicity of carboplatin plus paclitaxel combination therapy [127–129, 131], oxaliplatin [126], and cisplatin [133, 134], whereas two other studies have failed to show significant neuroprotection against carboplatin plus paclitaxel combination therapy [130] and cisplatin [132] (Table 2.3). BNP7787 (Dimesna, Tavocept, 2,29-dithio-bis-ethanesulfonate) has also shown some cytoprotective activities in vitro [174]. This effect was not confirmed in the clinical setting, although the study was unblinded with no placebo-controlled group and high risk of bias [135].

NGF Stimulants

Circulating nerve growth factor (NGF) levels are reduced in cancer patients with neuropathy caused by neurotoxic agents [175]. In addition, Schmidt et al. [176] showed in a mouse model that NGF exerted a major effect on the metabolism of transmitters associated with nociception, pain, and sensation in cervical dorsal root ganglia in various models of neurotoxicity, including the cisplatin-induced neuropathy. Thus, NGF in high doses may protect DRG neurons exposed to cisplatin [176], as well as against oxaliplatin-induced peripheral neurotoxicity [177]. They hypothesized that this effect could be due to NGF’s ability to preserve the correct neuronal differentiation status by blocking the cell cycle in the Go phase.

The synthesis of NGF can be stimulated by Org 2766 [34, 156, 159], leukemia inhibitory factor (rhuLIF) [162], retinoic acid [161, 178], glutamine [144], and acetyl-L-carnitine [179–181]. The two latter may also increase glutathione production [182, 183]. Two clinical trials [158, 159] with relatively small sample sizes showed some degree of Org 2766 neuroprotection in patients with peripheral neurotoxicity induced by cisplatin, whereas two other studies could not find a significant decrease in the incidence and severity of neuropathy [156, 157] (Table 2.3). Furthermore, derivatives of erythropoietin, a protein signaling cytokine, (e.g., carbamylated erythropoietin and asialo-erythropoietin) have been successful in vitro and in animal models [184, 185].

Retinoic acid (all-trans-retinoic acid) is a stimulator of NGF and the expression of its receptor, activator of retinoid acid receptors with neuroprotective profile [177]. It is also a prodifferentiating agent that counteracts platinum-induced neuronal apoptosis through activating both JNK/SapK and ERK1/2 [177]. Additionally, Arrieta et al. [186] reported a decrease in incidence and severity of neuropathy induced by cisplatin-paclitaxel combination when retinoic acid was administered.

Antioxidants or Antioxidant-Related Agents

α-Lipoic acid, a physiologic antioxidant with some neuroprotective activity [187], has recently been tested in a well-designed clinical trial [125] in which no significant decrease in incidence and severity of peripheral neurotoxicity induced by cisplatin and oxaliplatin has been reported (Table 2.3). α-Tocopherol (vitamin E), as another antioxidant, acts against free radicals. Four trials have evaluated the effect of vitamin E in preventing platinum, mainly cisplatin-induced peripheral neurotoxicity, and showed significantly lower incidence and severity of neuropathy in the vitamin E group compared with the control group [164–167], although all were with high risk of bias and low strength of evidence (Table 2.3).

Reduced glutathione is a natural neuroprotectant antioxidant derived from the γ-glutamyl transpeptidase with a high affinity for heavy metals, which may prevent the accumulation of platinum in the DRG [146, 147, 149, 150]. Additionally, it is a natural free-radical scavenger and can also stimulate NGF receptors [188]. Five different clinical trials demonstrated the potential of glutathione in reducing the incidence and severity of neuropathy induced by oxaliplatin [146, 147] or cisplatin [149, 150, 152],
whereas three others could not find significant neuroprotective effect against carboplatin (carboplatin-paclitaxel combination) [145] or cisplatin-induced peripheral neurotoxicity [148, 151] (Table 2.3). Similarly, oral glutamine, another derivative of the γ-glutamyl transpeptidase, may reduce the incidence and severity of oxaliplatin-induced peripheral neuropathy [144], although based on a randomized, but neither blinded nor placebo-controlled trial.

N-Acetylcysteine is a glutathione precursor that is believed to increase the blood concentration of glutathione [124]. The only available clinical trial on N-acetylcysteine in a small population revealed some potential neuroprotective effects against oxaliplatin-induced peripheral neurotoxicity [124] (Table 2.3). D-Methionine, a sulfur-containing nucleophilic antioxidant, has also shown successful neuroprotection against cisplatin-induced neurotoxicity in cortical network in vitro [189].

Electrolytes, chelators, and ion channel modulators

The electrolytes calcium and magnesium may act as chelators against oxalate accumulation and will probably protect the voltage-gated sodium channels from alteration [140]. Among six available randomized clinical trials conducted to evaluate the efficacy of calcium/magnesium infusion against oxaliplatin-induced peripheral neurotoxicity (Table 2.3), two preliminary studies were unsuccessful in showing any protection by intravenous calcium/magnesium [140, 141]. Knijn et al. [139], in a retrospective analysis study on patients with oxaliplatin-induced peripheral neurotoxicity, could only find reduced rate of grade 1 peripheral neurotoxicity, considering the high risk of bias. Later, two clinical studies have shown some levels of neuroprotection against the development of oxaliplatin-induced neuropathy [138]. However, the two most recent trials did not confirm the neuroprotective role of calcium/ magnesium against oxaliplatin-induced neuropathy [137, 136]. Moreover, Han et al. [136] have shown that calcium and magnesium infusions do not alter the pharmacokinetics of either intact oxaliplatin or free platinum, whereas there was no evidence of a pharmacokinetic interaction between calcium/ magnesium and oxaliplatin, meaning that these infusions may provide no benefit in reducing acute oxaliplatin-induced peripheral neurotoxicity.

Carbamazepine and oxcarbazepine are known as antiseizure drugs. They block voltage-sensitive sodium channels and some calcium channels, which might protect the voltage-gated sodium channels from alteration by oxalate [55, 190]. These two agents have also been tested in clinical trials with relatively small sample sizes, with one showing neutral effect [142] and the other suggesting benefit, although with an unblinded control arm [160] (Table 2.3).

Nimodipine is a calcium channel blocker that did not show significant neuroprotection against cisplatin in the only clinical trial ever done [155]. Although the trial had to be terminated because of severe gastrointestinal toxicity, the results by that time did not support neuroprotection. A multicenter trial on diethyldithiocarbamate, a chelating agent and antioxidant that prevents the degradation of extracellular matrix as an initial step in cancer metastasis and angiogenesis, did not demonstrate a significant chemoprotective effect against cisplatin-induced neurotoxicity [143] (Table 2.3).

Other compounds

There are also some data supporting the preventive effect of other agents against platinum-induced peripheral neurotoxicity. Acetyl-L-carnitine is a natural compound that plays a role in intermediary metabolism and has an antioxidant activity [191]. In vitro data support its effectiveness for platinum-induced neurotoxicity [179], but a recent randomized double-blinded placebo-controlled trial discouraged its administration for a non-platinum agent [192]. Xaliproden is a 5-hydroxytryptamine (HT)1A agonist that also acts as a neuromodulator with neurotrophic and neuroprotective effects in vitro [193] and had positive results in a clinical setting as well [168], although the results have yet to be published. Venlafaxine is a serotonin-norepinephrine reuptake inhibitor that also modulates the oxidative stress in the nervous system and may block sodium channels, which showed some neuroprotective effect in a small clinical trial [163].
Goshajinkigan (Kampo medicine), composed of 10 natural ingredients, is frequently used for alleviating symptoms of diabetic peripheral neuropathy in Japan; it is shown to have some neuroprotective potentials [194, 195]. Moreover, its safety and efficacy for preventing oxaliplatin-induced peripheral neurotoxicity have been tested in two clinical trials [153, 154] (Table 2.3). Nishioka et al. [154] reported a significantly lower incidence of grade 3 peripheral neurotoxicity, although based on a small sample size with unblinded control group. The findings of a phase II, multicenter, randomized, double-blind, placebo-controlled trial by Kono et al. [153] were also suggestive of reduced but insignificant rate of peripheral neurotoxicity grade 2 and 3 in patients treated with oxaliplatin compared with placebo (incidence of grade 2 neuropathy until the eighth cycle: 39% and 51% in the Kampo and placebo groups, respectively [relative risk, 0.76; 95% CI 0.47–1.21]; and grade 3: 7% versus 13% [0.51, 0.14–1.92]).

**Nondrug Approach**

There are other modalities that might enhance the effectiveness of the treatment while diminishing side effects or prevent peripheral neurotoxicity. It might be helpful to identify risk factors for neurotoxicity, such as pre-existing neuropathy, inherited neuropathies, age-related axonal loss, diabetes mellitus, alcohol abuse, and poor nutritional status, that may predispose to more severe symptoms from platinum-induced peripheral neurotoxicity [196].

Timing in drug administration to account for biological rhythms (chronotherapy) seems also very important [197], because there are drugs and disease conditions, including cancers suggestive of an optimal circadian time of drug administration [198]. However, in a meta-analysis on five randomized controlled trials with 958 patients, there was no significant difference in the incidence of peripheral sensory neuropathy after chronomodulation [199].

Finally, regarding the paucity of evidence about preventive and therapeutic strategies, treatment modification and drug withdrawal remain the most effective modalities for majority of patients [31], which indeed necessitates more adequately powered preclinical and clinical researches to find better alternative modalities [200, 201].

**Neuro- Versus Chemoprotection**

It is essential to demonstrate whether the application of a neuroprotective agent might diminish the efficacy of the therapeutic agent. However, this potential adverse effect has been tested with different agents. As an example, cisplatin and carboplatin are used in induction and myeloablative chemotherapy for high-risk neuroblastoma, but because of significant ototoxicity in children, their administration may be compromised. Harned et al. [169] showed that the exposure of six neuroblastoma cell lines to STS, at 6 hours after cisplatin, did not bind to and eliminate the circulating cytotoxic compound and thus did not affect the antitumor effect of the platinum agent, even under hypoxic conditions. However, a significant undesired protection against cisplatin cytotoxicity was seen when the neuroblastoma cells were simultaneously exposed to both cisplatin and STS combinations. Moreover, Harned et al. [169] demonstrated that in a subcutaneous neuroblastoma xenograft model in nu/nu mice, mice receiving cisplatin alone or cisplatin plus STS after 6 hours had significantly better progression-free survival rates (p < .03) compared with controls or mice treated with concurrent cisplatin and STS administration. Likewise, Muldoon et al. [170] reported that delaying the administration of STS for 6–8 hours after carboplatin did not reduce its antitumor activity in a human small cell lung cancer xenograft model in the rat, but still protected against ototoxicity in guinea pigs. Moreover, Dickey et al. [171] found that adding STS simultaneously or up to 2 hours postcisplatin protected against the antitumor effect of cisplatin in glioblastoma, SKOV3 ovarian carcinoma, medulloblastoma, and small cell lung cancer cell lines, but that delayed STS administration for 6 hours did not show a significant chemoprotection in any of the cell types.
The use of STS to prevent hearing loss in children with a variety of malignancies has been tested in two phase III randomized trials SIOPEL6 (NCT00652132) [172] and COG ACCL0431 (NCT00716976) [173]. In the preliminary report of COG, presented at the 2014 annual American Society of Clinical Oncology (ASCO) meeting [173], a protective effect of STS was found in reducing the proportion of hearing loss compared with observation (29% versus 55%; \( p = .006 \)). In this trial, 126 cancer patients were randomized to either cisplatin infusions alone or, to prevent cisplatin-induced hearing loss, combined with STS at 16 g/m2 IV over 15 minutes beginning 6 hours after the completion of each cisplatin dose. The median postdiagnosis follow-up was 2.1 years. However, the potentially lower survival seen in the patients with disseminated disease receiving STS (event-free survival 60% versus 70%, \( p = .53 \); overall survival 75% versus 89%, \( p = .50 \)) raises some concern of a tumor-protective effect of STS.

Treatment

The efficacy of some antidepressants, anticonvulsants, and a topical gel has been tested in six trials for treatment of platinum-induced peripheral neurotoxicity. Smith et al. [202] studied the effect of duloxetine in a randomized, placebo-controlled, crossover trial of 231 patients with either platinum or taxane neurotoxicity. Patients received 30 mg of duloxetine for the first week and 60 mg of daily duloxetine for 4 more weeks. Duloxetine significantly reduced pain and paresthesia, especially in oxaliplatin group. In contrast, 50 mg of daily amitriptyline or 100 mg of nortriptyline, in two separate trials, failed to demonstrate any significant improvements in patient-reported sensory symptoms, as well as objective scorings [203]. Similarly, trials testing gabapentin at a target dose of 2,700 mg/day [204] or lamotrigine at a target dose of 300 mg/day [205] failed to demonstrate any benefit for treatment of peripheral neurotoxicity. Finally, one trial evaluated a compounded topical gel containing baclofen (10 mg), amitriptyline HCl (40 mg), and ketamine (20 mg) on 208 randomly allocated patients [206], and a significant improvement in motor subscale scores was observed.

Future

There are some promising results favoring the ability of some neuroprotective agents to reduce the rate of subsequent neurotoxicity induced by platinum analogs. However, the most recent update of the Cochrane review on chemotherapeutic neuroprotective agents found insufficient data to conclude that any of the available chemoprotective agents is sufficiently effective in preventing or limiting the neurotoxicity of platinum drugs. Albers et al. [9] reviewed 29 randomized controlled trials (RCTs) or quasi-RCTs, in which 2,906 participants received chemotherapy with cisplatin or related compounds. Patients were also evaluated for quantitative sensory testing (primary outcome) or other measures including nerve conduction or neurological impairment rating using validated scales (secondary outcomes) before and 6 months after completing chemotherapy (Table 2.3). Likewise, the most recent ASCO Clinical Practice Guideline [123] based on a systematic review on 48 RCTs, including 35 RCTs on platinum-induced peripheral sensory neurotoxicity, did not recommend any established agent for the prevention of platinum-induced peripheral neurotoxicity. Only for the treatment of existing oxaliplatin neuropathy, they advised duloxetine, for which intermediate strength of evidence is present, considering the balance between benefit and harm [123].

Altogether, no neuroprotective strategy can yet be recommended for prevention and treatment of platinum-induced neurotoxicity, with a possible exception for duloxetine for oxaliplatin. There is a genetic diversity between patients, leading to differences in drug response including the side effects; hence a neuropathy preventive strategy should be individualized for each patient.

A pharmacogenetic approach might be useful in understanding the cause of peripheral neurotoxicity and tailoring the most suitable chemotherapy for each patient. A genome-wide pharmacogenomic approach may also be useful in identifying novel polymorphism predictors of severe platinum-induced peripheral neurotoxicity that may be used in personalized chemotherapy [71]. However, it is highly
recommended that the positive and negative effects of the antineoplastic agents be studied in detail in preclinical settings before implementation in clinical practice. In particular, the central nervous system of animals can be used to quantify the effects of platinated compounds on neurons that corroborate clinical data and suggest them as suitable models for studying possible neurotoxicity of platinum agents [111–121]. Some in vitro models can also be used to investigate morphological parameters affected by platinum compounds [122]. These models enable measuring the effect of the drugs on neurons along with testing the neurogenic potential of neuroprotective compounds.

**Conclusion**

Our knowledge about the pathophysiology of platinum-induced peripheral neurotoxicity and suggested neuroprotective strategies is diverse and not adequately powered. Therefore, a thorough investigation of available evidence is important to design new, solid studies to tailor appropriate treatment to individual patients. This will minimize the burden of peripheral neurotoxicity, optimizing the potentially positive impact of the chemotherapeutic medication.

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**Author Contributions**

Conception/Design: Abolfazl Avan, Tjeerd J. Postma, Godefridus J. Peters
Provision of study material or patients: Abolfazl Avan, Godefridus J. Peters
Collection and/or assembly of data: Abolfazl Avan
Data analysis and interpretation: Abolfazl Avan, Tjeerd J. Postma, Guido Cavaletti, Elisa Giovannetti
Manuscript writing: Abolfazl Avan, Tjeerd J. Postma, Cecilia Ceresa, Amir Avan, Guido Cavaletti, Elisa Giovannetti, Godefridus J. Peters
Final approval of manuscript: Abolfazl Avan, Tjeerd J. Postma, Guido Cavaletti, Elisa Giovannetti, Godefridus J. Peters

**Disclosures** The authors indicated no financial relationships.

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Characterization of and Protection from Neurotoxicity Induced by Oxaliplatin, Bortezomib and Epothilone-B

Cecilia Ceresa\textsuperscript{1,*}, Abolfazl Avan\textsuperscript{2,3,*}, Elisa Giovannetti\textsuperscript{3}, Albert A. Geldof\textsuperscript{4}, Amir Avan\textsuperscript{3}, Guido Cavaletti\textsuperscript{1}, Godefridus J Peters\textsuperscript{3#}

\textsuperscript{1}Department of Surgery and Interdisciplinary Medicine, University of Milano-Bicocca, Monza, Italy; \textsuperscript{2}Department of Neurology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran; \textsuperscript{3}Department of Medical Oncology, and \textsuperscript{4}Department of Urology, VU University Medical Center, Amsterdam, The Netherlands

(*These authors contributed equally to this study)
Oxaliplatin, bortezomib and epothilone-B induced neurotoxicity: characterization and protection

ABSTRACT: Aim: To characterize neurotoxicity induced by oxaliplatin, bortezomib, and epothilone-B as well as protection against their neurotoxicity using an in vitro model. Materials and Methods: Neurotoxicity was evaluated using the neurite outgrowth method in PC12 rat pheochromocytoma cells differentiated towards a mature neuronal phenotype, while neuroprotection was explored by simultaneous exposure to 0.5 mM amifostine. The potential markers of neuronal differentiation, cyclin-B2 (Ccnb2) and baculoviral inhibitor of apoptosis repeat-containing 5 (Birc5), were evaluated by quantitative reverse transcription polymerase chain reaction (RT-PCR). Results: Bortezomib, epothilone-B, and oxaliplatin reduced neurite length to 68%, 78% and 66%, respectively (p<0.05). The percentage of neurite-forming-cells (discriminating neurotoxicity from general cytotoxicity) decreased from 70% (control) to 55% (bortezomib), 46% (epothilone-B), and 51% (oxaliplatin). Amifostine was neuroprotective against oxaliplatin-induced neurotoxicity, increasing both neurite length and neurite-forming-cells. Quantitative-RT-PCR showed a 2.7-fold decrease in Ccnb2 expression in differentiated PC12 vs. undifferentiated cells. Conclusion: Oxaliplatin, bortezomib, and epothilone-B are neurotoxic in the PC12 model. Amifostine has a neuroprotective effect only against oxaliplatin-induced neurotoxicity, suggesting that these compounds have different mechanisms of neurotoxicity. Anticancer Res 2014;34(1):517-523

Key words: Neurotoxicity, oxaliplatin, amifostine, neuroprotection, PC12 model, Cyclin-B2

Introduction

Chemotherapy-induced peripheral neurotoxicity (CIPN) represents a clinically relevant problem in anticancer therapy. CIPN mainly presents with sensory symptoms, and is usually dose-dependent. It typically develops after a cumulative dose, even if acute toxic neuropathy is more common with some drugs (e.g. oxaliplatin and taxanes) (1). This side-effect might be dose-limiting or even lead to treatment withdrawal. However, even when neurotoxicity is not dose-limiting, it is an important side-effect that can irreversibly impair the patients’ quality of life by causing chronic discomfort (2-5). Not only established anticancer drugs, such as platinum compounds and taxanes, but also novel compounds, such as epothilones and bortezomib, have been reported to be neurotoxic, although the mechanisms of toxicity are likely to be different (6-8).

Bortezomib is a proteasome inhibitor registered for the treatment of multiple myeloma and mantle cell myeloma (9, 10), and is also tested against other diseases, usually in combination with platinated compounds (11). Peripheral neuropathy is among the most frequently-observed toxicities necessitating dose reductions or treatment discontinuation (11). At least one-third of patients under treatment have evidence of clinical sensory peripheral neuropathy (11-13). Although, it is not clear how and where bortezomib affects the peripheral nervous system, treatment withdrawal can result in clinical improvement of the induced neurotoxicity. As a result, further studies are essential in order to define the mechanism of this toxic effect, both alone and in combination with platinated compounds.

Epothilone-B is another new chemotherapeutic agent causing peripheral neurotoxicity due to its interaction with microtubule polymerisation inducing arrest in the G2/M transition (14), similarly to taxanes. Nevertheless, epothilones and taxanes differ in terms of resistance conferred by specific point-mutations in the gene encoding β-tubulin (15-17). Epothilones also induce dose-limiting peripheral neurotoxicity (18). Despite the fact that sensory peripheral neurotoxicity is regarded as a main toxic effect on the nervous system, motor or (rarely) autonomic neuropathy has occasionally been reported. Although no pathological data are available on epothilone-treated patients, dose-dependent axonal damage was demonstrated in animal models, in which recovery was obtained within few weeks after treatment withdrawal (19).
Oxaliplatin is a platinum-based compound with high efficacy in colorectal cancer, when administered in combination with 5-fluorouracil and folinic acid. Oxaliplatin exerts its cytotoxic effects through the formation of DNA adducts, with subsequent impairment of DNA replication and transcription, and causes severe and disabling sensory peripheral neurotoxicity due to accumulation of the drug in the dorsal root ganglia (DRG), where sensory neurons are located (20, 21).

The use of neuroprotective agents may help reduce neurotoxicity caused by chemotherapeutic agents, thus allowing for intensification of chemotherapy. One potential strategy in neuroprotection is the use of detoxicants such as amifostine. Amifostine is a phosphorylated amino-thiol pro-drug, which needs to be de-phosphorylated in order to exert its protective effect, which is selective in normal cells. Amifostine has been postulated to be selectively protective against normal tissue without reducing its antitumor activity (22-24). Amifostine is able to provide protection from the toxic effect of cisplatin in peripheral nerves (25-27).

Few pre-clinical models have been used to study the neurotoxic effects of novel chemotherapeutic drugs and neuroprotective agents (6, 28). Hence, the aims of the present study were: i) to characterize the extent of neurotoxicity induced by bortezomib, epothilone-B and oxaliplatin using the rat PC12 pheochromocytoma cell line to investigate the neurotoxic effects of different drugs; ii) to test the potential role of amifostine in the prevention of chemotherapy-induced neurotoxicity. In this model, neurotoxicity was assessed by standard quantitative morphological methods, including the counting of cells exhibiting neurites and the measurement of neurite length (28). Moreover, we evaluated the potential role of cyclin-B2 (Ccnb2) and baculoviral inhibitor of apoptosis repeat-containing 5 (Birc5) as surrogate biomarkers of neuronal differentiation using quantitative RT-PCR.

Materials and Methods

Drugs and chemicals. Bortezomib was a gift from Millenium Pharmaceuticals, Inc. (Johnson & Johnson, Cambridge, MA, USA), while oxaliplatin was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and epothilone-B from Calbiochem (Calbiochem, Darmstadt, Germany). Amifostine (S-2-(3-aminopropylamino)-ethylphosphorothioic acid, WR2721, Ethyol1) was obtained from USB Pharma (Nijmegen, the Netherlands). Epothilone-B and bortezomib were dissolved in dimethyl sulfoxide (DMSO), whereas oxaliplatin and amifostine were dissolved in sterile water and phosphate buffered saline (PBS), respectively. The drugs were diluted in culture medium before use. RPMI-1640 and Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were from Gibco (Gaithersburg, MD, USA). All other chemicals were from Sigma.

Cell culture. PC12 rat pheochromocytoma cells (29), were cultured in RPMI (containing 2 mM L-glutamine) supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, insulin-transferrin-sodium selenite medium supplement, penicillin (50 IU/ml) and streptomycin (50 μg/ml). Cells were maintained as monolayer cultures in 75 cm2 culture flasks (Costar, Cambridge, MA, USA), at 37°C in 5% CO2 and 95% air, and harvested with trypsin-EDTA when they were in exponential growth.

Cytotoxicity studies. The growth inhibition caused by bortezomib, epothilone-B and oxaliplatin on PC12 cells was evaluated with the sulforhodamine-B (SRB) assay (30, 31). For this purpose, cells were plated at a density of 20,000 cells/well in 96-well flat-bottom plates (Costar). Twenty-four hours later (day-0) cells were treated using different concentration ranges (0.0001-0.5 μM bortezomib, 0.0001-0.5 μM epothilone-B, and 0.05-100 μM oxaliplatin). The drug exposure time was 72 h (day 3). The IC50 and the IC80 were the drug concentrations at which cell-growth was inhibited to 50% and 80%, respectively, based on the difference of absorbance values on day 0 and day 3 of drug exposure, as calculated by the sigmoid inhibition model (GraphPad PRISM version 4.0; Intuitive Software for Science, San Diego, CA, USA).
**Neurotoxicity and PC12-neurite outgrowth assay.** The neurotoxic effects of bortezomib, epothilone-B and oxaliplatin were investigated using the nerve growth factor (NGF)-induced neurite outgrowth assay, as previously described (29, 32). Briefly, undifferentiated PC12 cells were pre-treated for five days by adding NGF 2.5S (NGF from mouse source; Promega, Madison, WI, USA) to a final concentration of 50 ng/ml, trypsinized, washed using PBS, and plated in 60-mm culture dishes (Costar) at a density of 25,000 cells/dish. Optimal adherence and neurite formation required precoating the plastic culture well using 0.5 mg/ml water solution of polylysine-hydrobromide (Sigma) followed by washing with sterile water. After 48 hours of culture with 10 ng/ml NGF, differentiated cells were exposed to bortezomib, epothilone-B or oxaliplatin at IC50 concentrations (0.95 nM bortezomib, 0.75 nM epothilone-B, 0.07 μM oxaliplatin). No drug was added to control dishes.

After 72 h of culture, the interference of cytostatic compounds on neurite outgrowth was evaluated by measuring the neurite length with an automatic image analyser (Quantimet, Leica, Cambridge, UK) on at least 200 randomly selected cells, and the percentage of differentiated cells per culture was calculated (33). For this purpose, cells were considered differentiated when the lengths of their neurite were longer than one-fold the cell body length (34). The morphometric determinations were always performed by the same examiner who was blinded to the treatment of the cell culture under observation. To discriminate specific neurotoxicity from general cytotoxicity, the analysis was performed on the basis of the fraction of differentiated cells instead of the absolute number of neurite-forming cells. The results, therefore, are given as the percentage of differentiated cells.

In order to evaluate the neuroprotective effect of amifostine, co-incubation experiments were carried out as follows. Differentiated PC12 cells were incubated with 0.5 mM amifostine and 2.7 nM bortezomib, 1.8 nM epothilone-B or 0.2 μM oxaliplatin. After 72 hours of culture cells were scored for neurites.

**Quantitative-RT-PCR.** Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was dissolved in RNase free-water, and measured at 260 nm. RNA (1 μg) was reverse-transcribed at 37°C for one hour in 100-μl reaction volume containing 0.8 mM dNTPs, 200 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT), 40 U of RNase inhibitor, and 0.05 μg/ml of random primers. The cDNA was amplified by quantitative-PCR with the Applied Biosystems 7500HT sequence detection system (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed in triplicate using 5 μl of cDNA, 12.5 μl of TaqMan Universal PCR Master Mix, 2.5 μl of probe and 2.5 μl of forward and reverse primers in a final volume of 25 μl. Samples were amplified using the following thermal profile: an initial incubation at 50°C for 5 min, to prevent the re-amplification of carryover-PCR products by AmpErase uracil-N-glycosylase (UNG), followed by incubation at 95°C for 10 min, to suppress AmpErase UNG activity and denature the DNA, 40 cycles of denaturation at 95°C for 15 sec followed by annealing and extension at 60°C for 1 min. Forward and reverse primers and probes for rat Ccnb2 (Rn01530826_g1) and rat glyceraldehyde 3-phosphate dehydrogenase (Gapdh, Rn99999916_s1) were obtained from TaqMan® Gene Expression Assays (Applied Biosystems), while forward and reverse primers and probes for rat Birc5 were obtained as custom TaqMan® Gene Expression Assays (Applied Biosystems), using the File Builder version 2.0 software, on the basis of GenBank database. Amplification data were normalized to the housekeeping gene Gapdh. Quantification of gene expression was performed using standard curves obtained with dilutions of rat cDNA from a mix of PC-12, BCLO, Bara-C and CC531 rat cell lines, and with dilutions of cDNA obtained from quantitative-PCR Human Reference Total RNA (Stratagene, La Jolla, CA, USA). PCR efficiencies were between 96.4 and 100%.

**Statistical evaluation.** All experiments were performed in triplicate and repeated at least three times. Data are expressed as mean values±SD and were analysed by ANOVA followed by the Tukey’s multiple comparison. Differences were considered significant when p<0.05.
Results

**Cytotoxicity experiments.** A concentration-dependent inhibition of cell growth was observed for PC12 cells. PC12 Cells were equally sensitive to bortezomib and epothilone-B (IC50 values of 0.9 and 0.7 nM, respectively). Oxaliplatin had a higher IC50 (70 nM). The IC50 of the single drugs was chosen to evaluate the neurotoxic effects, while the IC80 was used to evaluate the neuroprotective role of amifostine.

**In vitro neurotoxicity and protective role of amifostine.** The standard neurotoxicity assay measuring NGF-dependent neurite outgrowth from the PC12 pheocromocytoma cell line was used to predict neurotoxicity after 72 h incubation with bortezomib, epothilone-B, and oxaliplatin. Drug treatment of differentiated PC12 cells resulted in a statistically significant decrease of neurite length with respect to control cells. Bortezomib, epothilone-B, and oxaliplatin significantly reduced the neurite length to 68±5, 78±3, and 66±4%, respectively, compared to controls (Figure 3.1A). The percentages of cells expressing neurites, which were used to discriminate neurotoxicity from general cytotoxicity, also decreased significantly from ±3% in the control to 55±2% in bortezomib−, 49±3% in epothilone-B−, and 51±3% in oxaliplatin-treated cells (Figure 3.1B).

To investigate whether amifostine was able to prevent neurotoxicity induced by bortezomib, epothilone-B, and oxaliplatin in PC12 cells, the neurite length was also measured in PC12 cells exposed to amifostine for 72 h together with the single drugs, at their IC80. Amifostine partially reversed oxaliplatin-dependent neurotoxicity by reducing the shortening of neurite length from 59±3% (oxaliplatin alone) to 74±3% (oxaliplatin with amifostine). The percentage of neurite-forming cells increased from 42±5% to 59±3% for the combination. On the contrary, amifostine did not protect against the decrease of neurite length nor the percentage of neurite-forming cells induced by bortezomib and epothilone-B treatments (Figures 3.2A and B).

**Modulation of Ccnb2 and Birc5 mRNA by bortezomib, epothilone-B, and oxaliplatin.** Quantification of neurite outgrowth is the standard method to investigate neurotoxicity in vitro, but it is impractical on a large scale. For this reason, we examined changes in Ccnb2 and Birc5 mRNA expression in PC12 cells as potential biomarkers of neuronal differentiation. After differentiation towards a neuronal phenotype,
PC12 cells showed a 1.6-fold decrease in Ccnb2 expression compared to the undifferentiated control cells (Figure 3.3). Birc5 mRNA expression did not change (data not shown). The most neurotoxic drug in the neurite outgrowth assay, oxaliplatin, led to a 2.0-fold decrease in Ccnb2 expression at its IC50 (Figure 3.4), while bortezomib and epothilone-B induced a slighter modulation of Ccnb2.

![Figure 3.2](image)

**Figure 3.2.** Effect of amifostine (WR2721) on bortezomib, epothilone-B and oxaliplatin PC12 neurotoxicity in vitro. Nerve growth factor (NGF)-differentiated PC12 cells were exposed to 80% maximal-inhibitory concentrations (IC80s) of bortezomib, epothilone-B and oxaliplatin alone or in combination with amifostine (0.5 mM) for 72 h. The neurite length values (a) and the percentage of differentiated cells (b) were calculated as the percentage of values obtained in control cells. Columns, mean values obtained from three independent experiments; bars, SD. Significantly different from *control cells (p<0.001), #oxaliplatin-treated cells (p<0.01).

![Figure 3.3](image)

**Figure 3.3.** Effect of differentiation on cyclin B2 (CB2) level in PC12 cells. Modulation of CB2 expression in PC12 cells, as determined by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). CB2 expression was studied in nerve growth factor (NGF)-differentiated PC12 cells treated for 72 h with bortezomib, epothilone-B and oxaliplatin at their half-maximal inhibitory concentrations (IC50s) with respect to undifferentiated and NGF-differentiated PC12 cells. Columns, mean values obtained from three independent experiments calculated in comparison with standard curves and with respect to the respective expression values of the housekeeping gene Gapdh; bars, SE.

![Figure 3.4](image)

**Figure 3.4.** Effects of bortezomib, epothilone-B and oxaliplatin on cyclin-B2 levels in PC12 cells. Modulation of cyclin-B2 (CB2) expression as determined by real-time reverse transcription polymerase chain reaction (RT-PCR). CB2 expression was studied in nerve growth factor (NGF)-differentiated PC12 cells treated for 72 h with bortezomib, epothilone-B and oxaliplatin at their half-maximal inhibitory concentrations (IC50s) with respect to undifferentiated and NGF-differentiated PC12 cells. Columns, mean values obtained from three independent experiments calculated in comparison with standard curves and with respect to the respective expression values of the housekeeping gene Gapdh; bars, SE. Significantly different from *undifferentiated control cells (p<0.05).
Discussion

In the present study, the neurotoxic effects of bortezomib, epothilone-B and oxaliplatin were successfully evaluated in PC12 rat pheochromocytoma cells after neuronal differentiation. In agreement with clinical data, neurotoxicity induced by bortezomib, epothilone-B and oxaliplatin has been shown in our model, by reducing both neurite length and the percentage of neurite-forming cells. We also demonstrated the potential role of the expression of Ccnb2 mRNA as biomarkers of neuronal differentiation in PC12 rat cells. Moreover, amifostine protected against oxaliplatin-induced neuropathy with no adverse effect on treatment efficacy, but not against bortezomib and epothilone-B, suggesting a different mechanism of neurotoxicity.

Bortezomib-induced neurotoxicity was mainly characterized in rat models by axonopathy of unmyelinated fibres in nerves and pathological alteration in DRG satellite cells, while DRG neuron degeneration was observed only in mice (28, 35). Epothilone-B effects have not been studied extensively in pre-clinical settings. It is assumed that epothilone-B reduces the nerve conduction velocity (NCV), although no correlation has been found between the concentrations of the drug in tissues with NCV changes (35). Based on two studies, sensory neuropathy induced by ixabepilone (epothilone-B analog) varied from 3 to 59% (37, 38). Oxaliplatin-induced neurotoxicity is characterized by cumulative sensory neurotoxicity due to increased neuronal excitability by alteration of the voltage-gated sodium channels through chelation of calcium by the oxaliplatin metabolite (39). Further characterization of this induced neurotoxicity is crucial in order to find the most effective neuroprotective treatment and minimize the incidence and consequences of such a serious side-effect. Some agents such as vitamin E, carbamazepine, calcium and magnesium infusion, reduced glutathione, N-acetylcysteine and amifostine are considered as neuroprotective treatments, but they have not yet proven to be completely effective (1).

In the present work, we evaluated the potential neuroprotective effect of amifostine on bortezomib, epothilone-B, and oxaliplatin. Oxaliplatin-induced neurotoxicity was partially protected, by restoring neurite length and neurite-forming cells between 35-50% of the neurotoxicity induced by oxaliplatin alone. Importantly, in a clinical trial performed by Lu and colleagues, amifostine reduced the peripheral neurotoxicity due to oxaliplatin by about 60%, resulting in about 20% increase in the number of patients who received multiple chemotherapy cycles (40). The results obtained in our experimental setting thus confirm the reliability of the morphological method used in this study to test the potential neuroprotective activity of different drugs.

In order to evaluate potential biomarkers for neurite outgrowth, we also measured the mRNA expression of Ccnb2 and Birc5. Decreased levels of Ccnb2 and Birc5 have been correlated with neurite outgrowth induced by NGF in SH-SY5Y HN cells (41). Accordingly, our studies showed a significant reduction in Ccnb2 expression in NGF differentiated PC12 cells with respect to the control. Conversely, Birc5 mRNA level was not down-regulated following differentiation. Ccnb2 is an important regulator of the cell cycle and it associates with cyclin-dependent kinase 1 as its positive regulatory subunit. The decrease in Ccnb2 expression observed in PC12 cells following NGF differentiation is in accordance with previous data obtained in the same cellular model. These data suggested that the decrease in Ccnb2 expression after treatment with agents inducing neuronal differentiation could lead to cyclin-dependent kinase 1 inactivation with a consequent increase in neurite outgrowth (42, 43). The observed effect of oxaliplatin is probably due to reduction of cyclin-dependent kinase 1 activation induced by the antineoplastic drug with a consequent decrease in Ccnb2 expression, as observed in HCT116 colorectal cancer cells treated with oxaliplatin (44).

In conclusion, the experimental methods used in the present in vitro study, including a PCR analysis for Ccnb2 as a surrogate biomarker of neuronal differentiation, showed similar neurotoxic effects, especially for oxaliplatin. Moreover, we demonstrated that amifostine partially protects against oxaliplatin-induced neurotoxicity. These results prompt future pre-clinical and translational studies to test neurotoxicity of novel platinated compounds, as well as new neuroprotective agents, before starting the treatment.
References


Calcium/magnesium infusion for oxaliplatin-induced neuropathy: protective or not?

Abolfazl Avan*, Amir Avan¹, Elisa Giovannetti¹, Godefridus J Peters¹

¹VU University Medical Center, Amsterdam, the Netherlands
Calcium/magnesium infusion for oxaliplatin-induced neuropathy: protective or not?

TO THE EDITOR: Loprinzi et al [1] recently published results of a phase III trial showing that calcium/magnesium infusion does not decrease oxaliplatin-related neurotoxicity. This trial enrolled 353 patients with colon cancer who were undergoing adjuvant therapy containing oxaliplatin and were randomly assigned into three groups, including two groups who received calcium/magnesium according to different schedules. The authors used the sensory scale of the European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire–Chemotherapy-Induced Peripheral Neuropathy 20 tool (QLQ-CIPN20). This trial is of utmost importance because it might lead to a change in clinical practice. However, in our opinion, some key points should be discussed in more detail.

First, the authors [1] mentioned the subjective (patient reported) outcome measures as better tools for measuring symptoms. However, only a low correlation between all subjective and objective sensory grading scores was found in the cited study by Lavoie Smith et al [2]. Furthermore, the authors used two different versions of the Common Toxicity Criteria for validation (ie, versions 3 and 4). Nevertheless, in a recent study, the European Organisation for Research and Treatment of Cancer QLQ-CIPN20 sensory score was highly related to sensory items in Common Toxicity Criteria version 2.0 [3]. For now, no evidence demonstrates the superiority of a given method, whereas there are vast discrepancies between patients’ perception and objective tools, particularly in intermediate grades [3,4]. In addition, on the basis of data in Table 1 [1], the authors measured total QLQ-CIPN20 scores for sensory as well as autonomic and motor neuropathies. However, the correlations between autonomic and motor neuropathies and QLQ-CIPN20 scores are quite low [2,4], and additional information should be provided on how patients were educated to report these symptoms.

Second, the interpretation of prior beliefs with evidence is best achieved by Bayesian methods, not by Bonferroni adjustments, which are helpful in decision making but not in assessing evidence in data [5]. In particular, in the Bonferroni method, a universal null hypothesis must be postulated in which the two groups are identical with respect to all variables. This method will provide a correct answer if the variants are completely independent, but that is not the case in this study. Moreover, the likelihood of type II errors is inflated by this statistical analysis, so that truly important differences are deemed nonsignificant. Similarly, regarding the data in Table 1 [1], the Kruskal-Wallis test for nonordinal variables such as scorings and age, Fisher’s exact test for comparing four race/ethnicity subgroups, and the $\chi^2$ test for staging as a nominal variable should be replaced with more appropriate statistical methods [6].

Finally, according to the CONSORT statement [7], the authors should report a more detailed description of the methods and types of randomization and blinding they used in the trial, and should indicate whether those who assessed the outcomes were also blinded.

Overall, incorporation of the proper methodology in the conducting of the trial as well as in data analysis and reporting of results is essential to obtain solid evidence that can be used in clinical practice. Therefore, despite the importance of the trial conducted by Loprinzi et al [1], we believe that the question of whether calcium/magnesium infusion can protect against oxaliplatin-induced neuropathy is still open, and the results of this study need appropriate confirmation and replication.

Abolfazl Avan
VU University Medical Center, Amsterdam, the Netherlands

Amir Avan
VU University Medical Center, Amsterdam, the Netherlands; and Mashhad University of Medical Sciences, Mashhad, Iran

Elisa Giovannetti and Godefridus J. Peters
VU University Medical Center, Amsterdam, the Netherlands
AUTHORS’ DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

Correspondence to: Abolfazl Avan, MD; Department of Medical Oncology, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, the Netherlands. E-mail: abolfazl.avan@gmail.com

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PART 2
CHAPTER 5

Role of Akt signaling in resistance to DNA-targeted therapy

Abolfazl Avan¹, Ravi Narayan², Elisa Giovannetti¹, Godefridus J. Peters¹*

¹Department of Medical Oncology and ²Department of Radiation Oncology, VU University Medical Center, Amsterdam, The Netherlands.
Role of Akt signalling in resistance to DNA-targeted therapy

ABSTRACT: The Akt signal transduction pathway controls most hallmarks of cancer. Activation of the Akt cascade promotes a malignant phenotype and is also widely implicated in drug resistance. Therefore, the modulation of Akt activity is regarded as an attractive strategy to enhance the efficacy of cancer therapy and irradiation. This pathway consists of phosphatidylinositol 3 kinase (PI3K), mammalian target of rapamycin (mTOR), and the serine-threonine kinase Akt, also known as protein kinase B (PKB). DNA-targeted agents, such as platinum agents, taxanes, and antimetabolites, as well as radiation have a significant impact on cancer treatment by affecting DNA replication, which is aberrantly activated in malignancies. However, they may also trigger the activation of repair mechanisms, such as upstream and downstream cascade of Akt survival pathway. Thus, inhibition of this pathway can possibly improve the efficacy without compromising the effect of conventional treatment. Akt inhibitors, e.g. MK-2206 and perifosine, or PI3K modulators, e.g. LY294002 and wortmannin, have shown promising results in favor of sensitizing cancer cells to the therapy in vitro and in vivo, which have provided the rationale for incorporation of some of these inhibitors into multimodality treatment of different malignancies. Despite the acceptable safety profile of some of these agents in clinical studies the results are still too preliminary. Ongoing trials and translational studies will be critical to exploit these targeted compounds as well as to design more effective combination strategies with chemotherapeutic agents. Therefore, this review examines both the effects of different chemotherapeutic drugs on Akt signalling, and the effects of of Akt-inhibition on the efficacy of different anticancer agents.

Keywords: PI3K/Akt, platinum, taxane, antimetabolite, radiation.

1. Akt pathway signalling overview

The Akt signal transduction pathway plays a key role in the development of cancers, including metabolism, cell survival, cell cycle progression, regulation of gene expression, protein synthesis, motility, and genomic instability by phosphorylation of the substrates (reviewed in [1]). Aberrant loss or gain of Akt activation has been associated with the development of various diseases, e.g. diabetes, autoimmune diseases, and cancer [2-5].

The Akt pathway consists of phosphatidylinositol 3 kinase (PI3K), mammalian target of rapamycin (mTOR), and the transforming serine-threonine kinase Akt protein isoforms (further referred to as Akt), also known as protein kinase B (PKB) and phosphatase and tensin homologue (PTEN) as a critical tumor suppressor. PI3K enzymes phosphorylate phosphatidylinositol-4,5-biphosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP3) at the cell membrane that is required for the recruitment and activation of Akt. [6, 7] (Figure 5.1). These phospholipids are constitutively elevated in most cancer cells. Docking of Akt to the cell membrane causes a conformational change, which in turn leads to phosphorylation of the two critical amino acid residues, threonine 308 and serine 473, and finally leads to the activation of Akt [8]. After the activation, Akt is translocated to intracellular compartments where it phosphorylates several substrate proteins. The downstream targets of Akt are numerous due to the multiple interactions with its consensus sequence (1). In summary, the most important effects of Akt activation are: (a) cell survival through inhibition of BAD, caspase-9, and FOX [9-11]; (b) cell proliferation and gluconeogenesis through inhibition of GSK3, P11, P27, etc. [12]; and (c) protein synthesis and cell growth through activation of mTOR [13] (Figure 5.1).

To date, three Akt family members have been identified in mammals, i.e. Akt1 (also known as PKBα), Akt2 (PKBβ) and Akt3 (PKBγ). Having shown highly conserved properties, these homologues may be activated by the same mechanism [14]. However, being encoded by three different regions at 14q32, 19q13, and 1q44, respectively, these three isoforms are distinct substrates with distinct physiological outcomes, and also opposing to each other. Accumulating evidence puts Akt1 and Akt3 function almost
Phospho-Akt overexpression is prognostic and can be used to tailor the synergistic interaction of the novel Akt inhibitor perifosine with gemcitabine in pancreatic cancer

Amir Avan¹,³, * Mina Maftouh¹,* Abolfazl Avan¹,* Nicole van Grieken², Anne van Krieken¹, Rajiv Raktoe¹, Niccola Funel⁴, Kaamar Azijli¹, Sara Caponi⁵, Ugo Boggi⁶, Babette Aicher⁸, Leticia L. Leon⁹, Godefridus J. Peters¹, Elisa Giovannetti¹,⁷

Departments of ¹Medical Oncology and ³Pathology, VU University Medical Center, Amsterdam, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands, ³Department of New Sciences and Technology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran; Departments of ⁴Pathological Surgery, ⁵Oncology, ⁶General Surgery and ⁷Start-Up Unit University of Pisa, via Roma 55, 56100 Pisa, Italy; ⁸Æterna Zentaris GmbH, Weismuellerstrasse 50, 60314 Frankfurt am Main, Germany; ⁹Center for Biomedical Research of the Canary Islands, Instituto de Tecnologias Biomedicas, University of La Laguna, La Laguna, Spain.

*These authors equally contributed to this work
Phospho-Akt overexpression is prognostic and can be used to tailor the synergistic interaction of the novel Akt inhibitor perifosine with gemcitabine in pancreatic cancer.

**ABSTRACT:** There is increasing evidence of a constitutive activation of Akt in pancreatic ductal adenocarcinoma (PDAC), associated with poor prognosis and chemoresistance. Therefore, we evaluated the expression of phospho-Akt in PDAC tissues and cells, and investigated the therapeutic potential of the novel Akt inhibitor perifosine in combination with gemcitabine in PDAC cells. Phospho-Akt was overexpressed in 60% of PDACs as determined by immunohistochemistry, which was correlated with shorter survival in 50 resected PDAC patients. mRNA and protein expression levels varied considerably in 14 PDAC cells, as assessed by RT-PCR, immunocytochemistry and ELISA. Perifosine inhibited cell growth in monolayer cell cultures and spheroids, and synergistically enhanced the antiproliferative activity of gemcitabine, with combination index values of 0.4 and 0.8 in cells characterized by high phospho-Akt expression (LPC028 and CFPAC-1), while this combination was antagonistic in LPC006 cells, with low phospho-Akt expression. The synergistic effect was associated with reduction of the expression of the ribonucleotide reductase subunit-M2, potentially facilitating gemcitabine cytotoxicity. Moreover, perifosine decreased cell migration and invasion, which was additionally reduced by the perifosine/gemcitabine combination. The combination enhanced the percentages of cells in S-phase, and significantly increased apoptosis, associated with induction of caspase-3/6/8/9, PARP and BAD, and inhibition of Bcl-2 and NF-κB. In summary, these data provide novel insights into the ability of perifosine to interfere with cell proliferation, induce apoptosis, reduce migration/invasion and synergistically interact with gemcitabine in cells with phospho-Akt overexpression. Furthermore, these results support the analysis of phospho-Akt expression as a biomarker for the rational development of this innovative therapeutic approach.

Keywords: Pancreatic ductal adenocarcinoma, Akt, perifosine, gemcitabine

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal solid tumors. Despite extensive preclinical and clinical research, the prognosis of this disease has not improved, with a 5-year survival rate below 5% [1]. This dismal outcome can partially be explained by the lack of biomarkers for screening and diagnosis at earlier stages, and by the resistance to currently available chemotherapy regimens [2].

Activating KRAS mutations occur early, followed by loss of p16, and then later, inactivation of TP53 and SMADs [3]; however, targeting these events has proven to be very difficult. Studies on new drugs targeting additional oncogenic pathways and/or downstream effectors are warranted. The phosphatidylinositol-3 kinase (PI3K)/Akt pathway emerged as one of the core signaling pathways in PDAC [4]. In particular, the serine/threonine kinase Akt, which is coded in three highly homologous isoforms (Akt1, Akt2, and Akt3), is overexpressed in more than 40% of PDAC patients [5,6]. Mechanisms underlying aberrant Akt activation in cancer include direct alterations such as mutations, amplification, or overexpression, as well as also activation of upstream signaling events, such as activation of HER-signal, signaling or PTEN mutations/losses [7].

The PI3K/Akt pathway plays a key role in cell proliferation, survival and motility [8]. Deregulation of components involved in this pathway could confer resistance to chemotherapy [9,10], while blockade of Akt signaling result in programmed cell death and inhibition of tumor growth [11].

Activation of Akt is a frequent event in PDAC and has been correlated to its poor prognosis [12,13]. Foy and collaborators showed that inhibition of the PI3K/Akt pathway sensitizes pancreatic cancer cells by increasing apoptosis both in vitro and in vivo [14].
CHAPTER 7

Predictive role of repair enzymes in the efficacy of cisplatin combinations in pancreatic and lung cancer

Godefridus J Peters¹, Abolfazl Avan¹, Marielle Gallegos Ruiz, Vanessa Orsini¹, Amir Avan¹,², Elisa Giovannetti¹, Egbert F. Smit³

Departments of ¹Medical Oncology and ³Pulmonology, VU University Medical Center, Amsterdam, the Netherlands; ²Cardiovascular Research Center and Department of New Sciences and Technology, School of Medicine, Mashhad University of Medical Science, Mashhad, Iran

Predictive role of repair enzymes in the efficacy of cisplatin combinations in pancreatic and lung cancers

**ABSTRACT:** Platinum combinations are the mainstay of treatment for non-small cell lung cancer (NSCLC), while for pancreatic cancer platinum combinations are being given to good- performance status patients. These platinum combinations consist of cis- or carboplatin with gemcitabine, while, for non- squamous NSCLC and mesothelioma, of pemetrexed. The combination of gemcitabine and cisplatin is based on gemcitabine-induced increased formation and retention of DNA–platinum adducts, which can be explained by a decrease of excision repair cross-complementing group-1 (ERCC1)-mediated DNA repair. In these patients, survival and response is prolonged when ERCC1 has a low protein or mRNA expression. A low expression of ribonucleotide reductase (RR) is related to a better treatment outcome after both gemcitabine and gemcitabine- platinum combinations. For pemetrexed combinations, ERCC1 expression was not related to survival. For both NSCLC and pancreatic cancer, polymorphisms in ERCC1 (C118T) and Xeroderma pigmentosum group D (XPD) (A751C) were related to survival. In currently ongoing and future prospective studies, patients should be selected based on their DNA repair status, but it still has to be determined whether this should be by immunohistochemistry, mRNA expression, or a polymorphism. Anticancer Res 2014;34(1):435-442

**Key Words:** Non-small cell lung cancer, pancreatic cancer, gemcitabine, cisplatin, DNA repair, ERCC1, XPD, ribonucleotide reductase, review.

**Introduction**

Non-small cell lung cancer (NSCLC) and pancreatic ductal adenocarcinoma (PDAC) account for approximately 85% of lung and pancreatic cancer, respectively, and have a 5-year survival of less than 5% (1). Platinum combinations are standard therapy for both NSCLC and malignant pleural mesothelioma (MPM) but are still experimental in PDAC (2). Pemetrexed-platinum therapy is standard for MPM and non- squamous NSCLC, while for squamous NSCLC, gemcitabine-cisplatin is often used (3). The combination of raltitrexed and cisplatin was as similarly active in MPM as pemetrexed–cisplatin or pemetrexed–carboplatin (4).

All combinations are based on the assumption that in the combination, DNA damage is more extensive and repair is inhibited. Among the various DNA repair systems, the nucleotide excision repair (NER) system seems the major system involved in the repair of DNA–platinum adducts (5, 6), although the mismatch repair system also plays a role for cisplatin and carboplatin, but not for oxaliplatin (7). Several proteins are involved in the recognition of DNA damage, unwinding, subsequent excision of the damaged nucleotides (AG and GG Pt–adducts), and insertion of new deoxynucleoside triphosphates (dTTP) in the DNA. These include transcription factor II H (TFIIH), the xeroderma pigmentosum group enzymes XPD, XPC, ERCC1/XPF, DNA polymerases δ and ε, and ligase 1 (Figure 7.1). Single nucleotide polymorphisms (SNP) in any of these genes may affect the repair capacity and contribute to individual variations in chemotherapy response. Hence these repair systems have been extensively been investigated as an explanation for the interaction of gemcitabine with cisplatin (8, 9). Such pharmacogenetic studies may allow the variation in how individual patients respond to medicines to be reduced by tailoring therapies to their genetic profile (10).

**Pre-clinical Basis for The Interaction of Gemcitabine and Cisplatin**

The molecular basis for the interaction between cisplatin and gemcitabine was initially investigated in cell lines of ovarian, head and neck, and NSCLC (11-13). In these model systems, we demonstrated one of the most pronounced synergisms between two drugs, with combination indices (CI) of 0.001 and less (11). The combination was also more than additive in in vivo tumors generated from these cell lines (12-14); the most active schedule consisted of every 3-day gemcitabine schedule (four times), combined
we demonstrated one of the most pronounced synergisms followed by ligation via DNA ligase to complete NER and form a DNA as a template to synthesize a short complementary sequence, nucleotides. DNA polymerase then uses the undamaged single-stranded endonuclease which cuts DNA damage on the 3’ side while the XPF-are made both up- and downstream of the lesion by XPG and XPF- and XPB/C act as a helicase and ATPase, respectively. Next incisions Xeroderma pigmentosum group D (XPD). The TFIIH subunits of XPD unwound by transcription factor II H (TFIIH), which is stabilized by Xeroderma pigmentosum group D (XPD).

Figure 7.2. Correlation between Pt–DNA adduct formation and retention (expressed as area under the curve of adducts) and sensitivity of Lewis lung tumors to various gemcitabine–cisplatin treatments. The treatment with the highest extent of adducts was also the most effective treatment (Van Moorsel et al. (15), with permission).

Figure 7.1. Schematic action of the nucleotide excision repair (NER) pathway. In NER, after DNA damage is recognized, the DNA helix is unwound by transcription factor II H (TFIIH), which is stabilized by Xeroderma pigmentosum group D (XPD). The TFIIH subunits of XPD and XPB/C act as a helicase and ATPase, respectively. Next incisions are made both up- and downstream of the lesion by XPG and XPF- excision repair cross-complementing group 1 (ERCC1). XPG acts as an endonuclease which cuts DNA damage on the 3’ side while the XPF-ERCC1 heterodimeric protein cuts on the 5’ side. The dual incision leads to the removal of a ssDNA with a single-strand gap of 25-30 nucleotides. DNA polymerase then uses the undamaged single-stranded DNA as a template to synthesize a short complementary sequence, followed by ligation via DNA ligase to complete NER and form a double-stranded DNA. Partially modified from (6).

with cisplatin only on the first day. Both gemcitabine-preceding-cisplatin and cisplatin preceding gemcitabine were very active, although the gemcitabine preceding cisplatin became the most widely used schedule. Detailed analysis of the molecular interaction demonstrated that gemcitabine increased the formation of cisplatin–DNA adducts (13-16), both in vitro and in vivo. A detailed analysis in murine Lewis lung with NSCLC showed that Pt–DNA adduct levels (especially Pt–GG), evaluated as the areas under the concentration–time curves of Pt–DNA in the tumors, were associated with reduced doubling-time of tumors treated with the combination and this was related to the antitumor effect (Figure 7.2). In vitro it was also demonstrated that cisplatin increased the incorporation of gemcitabine into DNA (13). Most likely this increased incorporation was responsible for a structural change in the DNA, allowing more DNA adducts to be formed; this change might also affect inhibition of DNA repair. The extent of ERCC-mediated DNA repair in the gemcitabine–cisplatin combination was correlated with the extent of synergism (17). Selvakumaran et al. demonstrated in an in vivo system with ERCC1 antisense transfectants that ERCC1 is essential for in vivo repair of DNA platinum adducts (18).

For the repair of DNA damage, DNA polymerases require a sufficient supply of dNTP which are provided by the action of ribonucleotide reductase (RR), which has two subunits RRM1 and RRM2.
Inhibition of RR by gemcitabine reduces the concentration of dATP, dGTP and dCTP (19), this decrease hampers the repair of both AG and GG adducts. Hence RR expression is likely to play a role in the repair of Pt–DNA adducts, as outlined below.

Genetic and epigenetic alterations, such as gene mutations, amplification, deletions, polymorphic status, or altered gene/protein expression have been shown to be correlated with drug responses, including of gemcitabine and platinum analogs (6, 8, 20). Among the possible predictive or prognostic factors of survival benefit to a specific treatment, germline polymorphisms have been identified as an attractive target, specifically for advanced cancer, since their analysis can be more easily performed compared to tumor mutational analysis and gene expression arrays (21). Therefore, in the following, we mainly focused on gene expression alterations or polymorphic status of the genes involved in DNA repair systems and drug metabolism.

**ERCC1 Expression and Repair of Pt–DNA Adducts in Lung Cancer**

Since its introduction into the clinic, the cisplatin–gemcitabine combination is still considered a standard regimen for the treatment of advanced NSCLC (22). In a randomized four-arm phase III study, no difference was observed in time-to-progression (TTP), and overall survival (OS) between paclitaxel–cisplatin, docetaxel–cisplatin, paclitaxel–carboplatin, and gemcitabine–cisplatin combinations (2) and in a three-arm study between paclitaxel-cisplatin, gemcitabine-cisplatin or paclitaxel–gemcitabine (23). Because of better tolerance, the gemcitabine–cisplatin (or carboplatin) regimen is still considered as the standard regimen, although pemetrexed has replaced gemcitabine for non-squamous NSCLC based on a phase III study (3).

In an initial study by Lord et al. (24), it was demonstrated that patients with NSCLC with a low ERCC1 mRNA expression (as determined by PCR) had increased survival compared to those with high ERCC1 expression. In subsequent analyses both mRNA expression by PCR and immunohistochemistry were used to determine the expression of ERCC1. Although some antibodies against ERCC1 recognized another protein (25-27), a meta-analysis from 12 studies and 836 patients clearly demonstrated that low levels of ERCC1 mRNA or protein expression were associated with a longer survival [odds ratio (OR)=0.77, 95% confidence interval (CI)=0.47-1.07, \( p < 0.00001 \)] and a superior major response rate (OR=0.48, 95% CI=0.35-0.64, \( p < 0.00001 \)) (28). An even stronger correlation was observed when different parameters were combined. Ceppi et al. (29) demonstrated that a low expression of ERCC1 or RRM1 each were associated with a longer survival for patients treated with gemcitabine–cisplatin (ERCC1 p=0.0032; RRM1 p=0.039) but when the two were combined (both low), the survival benefit increased and was more significant (\( p = 0.0023 \)); similar results were observed by Bepler et al. (30). This information was recently applied in order to select patients on the treatment arm most likely to be sensitive to these combinations (31); from 275 eligible patients, those with a low RRM1/ERCC1 expression were randomized for the gemcitabine–carboplatin combination, those with a high RRM1 and low ERCC1 for docetaxel–cisplatin, and those with a high RRM1 and high ERCC1 for docetaxel–vinorelbine. No statistically significant differences were observed between the experimental and control arms regarding progression-free survival (6.1 vs. 6.9 months) and overall survival (11.0 vs. 11.3 months). However, all patients who received the same treatment and had a low expression of ERCC1/RRM1 had better progression-free survival (8.1 months) in the control group, compared to the experimental arm (5.0 months). Another major conclusion of this study was that measurement of protein expression was feasible and very reproducible.

Both immunohistochemistry and PCR analysis may have problems regarding their use in large groups of patients, since antibodies have to be validated (which is not always done properly) and may change in time (25-27), while sufficient RNA cannot always be isolated from tumor samples, albeit the technology to isolate sufficient and high quality RNA from paraffin-embedded tissues has improved considerably in the last decade. We compared immunohistochemistry, PCR and genetic polymorphisms for several biomarkers for their potential in patients with MPM (32). Indeed immunohistochemistry
proved to be too variable to draw firm conclusions on the ERCC1 expression in this cohort of patients.

In NSCLC, we found a high expression in cytoplasm, which was associated with a longer survival, a finding which seems counterintuitive, but this might be explained by the localization (Figure 7.3a).

An in vitro analysis demonstrated that the C/T SNP at codon 118 of the ERCC1 gene could have an influence on mRNA and protein levels (33), while some clinical data support a possible correlation of this SNP with survival in advanced NSCLC being treated with platinum-based chemotherapy (34, 35). Therefore we analyzed this polymorphism in our patients. In this group of patients, we observed that the ERCC1 C118T SNP was associated with a larger number of responding patients (Figure 7.3b).
Expression of ERCC1 was also related to the C118T SNP; in cytoplasm of tumor cells of patients with a TT genotype, there was a low intensity (0-1), in those with a CT genotype a two-fold higher intensity (0-2). In the nuclei of tumor cells of patients with a TT genotype, there was a high intensity (0-6), but in those with a CT genotype a lower intensity (0-4) (Figure 7.3c-d). Another intriguing finding in this cohort of patients was the association of an XPD polymorphism (A751C lys-gln) with longer survival of these patients (Figure 7.4). For the pemetrexed–carboplatin combination A751 gln-gln was associated with a shorter survival (36). Another polymorphisms of XPD (Asp312Asn) was associated with longer survival in patients treated with a carboplatin–taxane combination (37), as well as NSCLC (38). It can be concluded that in NSCLC, ERCC1 and RRM1 expression and ERCC1 and XPD polymorphisms may be associated with response to cisplatin therapy. However, a recent meta-analysis concluded that the predictive value of ERCC1 and XPD polymorphisms in patients with advanced NSCLC receiving platinum-based chemotherapy may both be important (39). The discrepancies observed among the studies may be due to differential methods, treatment heterogeneity, and relatively small sample size.

Triggered by the analysis of various DNA repair systems in the process of DNA platinum adduct repair, we analyzed these parameters in MPM, also treated with a platinum combination. The standard treatment for MPM is a combination of the antifolate pemetrexed and cisplatin or carboplatin. Pemetrexed is a multi-targeting antifolate, which not only inhibits its major target thymidylate synthase, but also dihydrofolate reductase, as well de novo purine nucleotide synthesis. Inhibition of thymidylate synthase will lead to a decrease in dTTP, which will affect DNA repair, but differently compared to inhibition of RRM1. In this study, a low thymidylate synthase expression was associated with a longer survival (18.0 months) compared to those patients who has a high expression (9 months, \( p=0.022 \)) (32). In the same cohort of patients, ERCC1 expression by immunohistochemistry failed to show any significant association, with an overall survival of 12 months for the low expression and 18 for the higher expression groups.

**The Role of DNA Repair in PDAC**

Standard treatment in PDAC is gemcitabine, which replaced 5-fluorouracil (5FU) in the late 1990s. From all combinations which have been investigated, the platinum combinations seemed most promising, in a setting either with cisplatin or oxaliplatin (40, 41). The combination treatment of FOLFIRINOX (5FU with leucovorin, irinotecan and oxaliplatin) produced the best results in patients with a good performance status who were able to tolerate this potentially toxic regimen. An alternative, the oral 5FU formulation S-1 with cisplatin produced a longer survival in patients with the ERCC1 C1187 genotype (CT and TT; \( p=0.030 \)) (42). In an analysis of 122 patients, treatment with gemcitabine-based polychemotherapy, the TT genotype was associated with a 13.3-month median survival (95% CI=9.7-17.0) compared to CC+TT (11.8 months, 95% CI=10.4-13.4; \( p=0.44 \)) (43). For XPD, an association between polymorphisms was found for both the XPD Asp312Asn and XPD lys-751gln. The Asn-Asn genotype was associated with a shorter overall survival (11.2 months, 95% CI=10.9-15.7 months)
compared to the Asp-Asp phenotype (15.1 months, 95% CI=10.4-19.4 months; p=0.010). Similarly the XPD GLn751Gln had a shorter survival of 10.3 (95% CI=4.0-16.5) months compared to the LysLys + Lys+Gln cohort (13.3 months, 95% CI=10.9-15.7; p=0.003). Survival of those with XPD Gln751Gln was shorter compared to those with other genotypes. In a larger cohort, 247 patients were treated with multiple drug combinations (PEXG: gemcitabine-cisplatin + epirubicin- xeloda; PDXG: gemcitabine-cisplatin + docetaxel-xeloda; EC-Gem-Cap: gemcitabine-cisplatin (i.a.) + Epirubicin (i.a.)- xeloda (i.a.) or gemcitabine alone (90 patients). In this polychemotherapy schedule, the XPD GLn751Gln conferred a poor survival (Figure 7.5a) (44). In this study, the genetic polymorphisms were also investigated for a functional association assuming that a decreased repair ability is related with the formation of more Pt−DNA adducts; indeed, white blood cells with the specific genotype (Lys751Lys) have a lower repair capacity compared with cells with Gln751Gln); this formation of Pt−DNA adducts was enhanced by gemcitabine in the Lys751Lys cohort (Figure 7.5b). Hence, the analysis of the polymorphism by a simple blood test offers an innovative tool for optimizing palliative chemotherapy in patients with advanced PDAC.

![Graph](image)

**Figure 7.5. A:** Association of the Xeroderma pigmentosum group D (XPD) A751C genotype with overall survival of patients with pancreatic ductal adenocarcinoma (PDAC) (213 patients with XPD Lys751Lys, or XPD Lys751Gln and 33 with XPD Gln751Gln, median overall survival 13.0 (11.4-14.6) and 7.0 (4.0-10.0) months) treated with gemcitabine- and cisplatin-containing polychemotherapy regimen (44). From (44) with permission. **B:** White blood samples from five volunteers per group A751A, A751C and C751C were tested for their DNA repair ability after exposure to 1 μM gemcitabine, 200 μM cisplatin or the combination for 24 h, after which a target sequence of β-globin was amplified with extra-long PCR (XL-PCR) as described earlier (44). The extent of DNA repair ability was based on the reduction of PCR amplification of the target sequence. Data are means±SD. Significantly different from *XPD Gln751Gln, **XPD Gln751Gln, or XPD Lys751Gln.

**Conclusion**

The efficacy of platinum combinations is dependent on the formation of platinum adducts. The extent of their formation and retention are controlled by DNA repair enzymes, but the contribution of each enzyme may be different for various diseases and combinations. For example, in a recent study in 19 head and neck cancer cell lines, the sensitivity to cisplatin was associated with DNA adduct formation and retention but not with the expression of any repair enzyme or other potential markers of resistance, such as drug transporters, which were shown to play a role in cisplatin resistance (45).
seems that in NSCLC treated with gemcitabine–cisplatin combinations, ERCC1 expression is related to efficacy, but this does not hold true for pemetrexed combinations in MPM.

Another major problem is the method used to determine the expression of the mRNA the protein. Although immunohistochemistry is a sensitive and versatile method for determination of protein expression, it is largely empirical, and the outcome mainly depends on which antibody is being used and also on the pathologist’s expertise. A major disadvantage of protein expression is the potential lack of specificity of antibodies (26, 27), potentially leading to incorrect expression data. Another problem is the cellular distribution of the protein, as shown for the cytoplasmic and nuclear staining (Figure 7.3). Hence new antibodies should be characterized thoroughly, preferably in model systems lacking or having high expression. Several studies have used the quantitative RT-PCR technique, but mRNA expression can differ from protein expression. mRNA isolation was considered a problem, but nowadays mRNA can be isolated in reliable quantities, even from paraffin-embedded tissues. The disadvantage is the lack of pathological confirmation of the sample, although RNA can be isolated from tumor-enriched parts, or one can use laser microdissection. Therefore, optimization and standardization of these two modalities with appropriate controls, which can be used for inter-laboratory validation, are essential before larger prospective investigations in homogeneously treated patients, which can address the same pharmacogenetic question.

From the same sample, DNA can be isolated for SNP analysis. Although SNP analysis using DNA is much more reliable, most data are not strong enough (small groups) to use in prospective studies. These studies are essential to select those patients who are likely to respond to the standard treatment and to select patients who are eligible for therapy with tyrosine kinase inhibitors such as erlotinib and gefitinib for patients with activating mutations for EGFR, or with crizotinib for patients with ALK expression (46). Since many of these tyrosine kinase inhibitors are synergistic with DNA-targeted therapy, such as gemcitabine–cisplatin or pemetrexed–cisplatin, pre-treatment analysis of gene expression or SNP will help select the treatment most likely to be effective.

Further investigations are needed to evaluate these emerging biomarkers, as well as to identify and select the optimal patient populations that will benefit from specific treatments. Together with the standardized techniques for sample collection and processing, larger and uniformly treated populations, and integration with functional data are necessary in order to validate the best markers for personalized treatment of patients.

Correspondence to: Godefridus J. Peters, PhD, Department of Medical Oncology, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, the Netherlands. Tel: +31 204442633, e-mail: gj.peters@vumc.nl

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CHAPTER 8

Modulation of Signaling Enhances the Efficacy of the Combination of Satraplatin and Erlotinib

Abolfazl Avan,1 Auke D. Adema,1 Eveline K. Hoebe,1 Charlotte M. Huijts,1 Amir Avan,1,2 Gareth J. Veal,3 R. Ruitenbeek,4 K. Wosikowski,5 Godefridus J. Peters1*

1Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands; 2Department of New Sciences and Technology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran; 3Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, United Kingdom; 4Pamgene, ‘s Hertogenbosch, the Netherlands; 5Previously at GPC Biotech, Munich, Germany; current address: Isarna Therapeutics, Munich, Germany
Modulation of signaling enhances the efficacy of the combination of satraplatin and erlotinib

Abstract: The active metabolite (JM118) of the oral platinum analog satraplatin (JM216) was investigated for potential synergism with erlotinib, an epidermal growth factor receptor (EGFR) inhibitor. JM118 sensitivity of 7 cancer cell lines (ovarian: 2008, A2780; colon: LoVo92, WiDr; lung: A549, SW1573; epidermoid: A431), was enhanced most pronounced when JM118 preceded erlotinib, which was associated with increased formation of DNA-platinum adducts. The combination increased G2/M phase accumulation and enhanced apoptosis. JM118 increased the phosphorylation of the cell cycle proteins CDK2 and CHK1 after 24 hr exposure. JM118/erlotinib enhanced Erk and Akt phosphorylation after 2 hr. JM118 significantly decreased the phosphorylation of PTEN, VEGFR, EPHA1, ERBB4, FGFR, and STAT3 by 20 (PTEN) to >90% (STAT3). Conclusion: Erlotinib enhanced the effects of JM118, even in cells with mutations in Ras. The mechanism of synergy involved a combination of effects on platinum-DNA adduct formation, cell cycle distribution and signaling. Curr Drug Targets 2014;15(14):1312-1321

Keywords: Satraplatin, erlotinib, signaling, Akt, Erk, colon cancer, lung cancer

Introduction

Platinum compounds are the mainstay of treatment of various malignancies. Cisplatin is used for the treatment of cancers such as lung, cervical, ovarian, testicular and squamous cell carcinoma of the head and neck [1], while oxaliplatin is mainly used for the treatment of colorectal cancers [2]. Platinum compounds are always given parenterally, usually in combination with other drugs such as gemcitabine, taxanes, or 5-fluorouracil (5-FU) [3-5]. Platinum derivatives form adducts with DNA [6], which is sensed as damage by repair pathways and will either be repaired, or the cells will enter the apoptosis pathway [7]. Cisplatin-DNA adducts can be recognized by the mismatch repair pathway or the nucleotide excision repair pathway, while oxaliplatin-DNA adducts are only recognized by the nucleotide excision repair pathway [3, 8].

Satraplatin (JM216) is the first oral platinum analog [9], exhibiting greater lipophilicity compared to carboplatin and cisplatin [10], favorable bioavailability [9, 11, 12], effective penetration through the blood brain barrier [10] and a good safety profile lacking nephrotoxicity and neurotoxicity compared to cisplatin in vivo [13, 14].

Adduct formation by satraplatin is mostly comparable to cisplatin, since when JM118 loses its acetate group it is structurally similar to cisplatin [15]. Nevertheless, satraplatin compounds have much higher potency compared to cisplatin [16], possibly due to DNA replication and transcription, leading to cell cycle arrest in the G2 phase and finally apoptosis [17]. Moreover, adducts formed by satraplatin are bulkier, more efficient in inhibiting DNA synthesis and less recognizable by DNA mismatch repair system compared to cisplatin and carboplatin [8, 18].

Satraplatin has shown some efficacy in Phase II studies with chemo naive small-cell lung cancer patients and advanced non-small cell lung cancer (NSCLC) [19, 20] as well as cervical cancer, hormone refractory prostate cancer and breast cancer [21-23]. More promising efficacy was found in a combination of satraplatin plus prednisone versus prednisone alone in patients with castrate- resistant prostate cancer [24], with an increased median progression-free survival (PFS) (5.2 vs 2.5 months; \(p = .023\)) compared to the prednisone-alone arm. However, in a larger Phase III trial (Satraplatin and Prednisone Against Refractory Prostate Cancer [SPARC]) with 950 pretreated prostate cancer patients, despite the mild toxicity along with improved PFS and the palliative effects, satraplatin plus prednisolone failed to demonstrate a significant improvement in overall survival [25].

Satraplatin has also been tested in combination with bevacizumab, a monoclonal antibody against vascular endothelial growth factor, [26] with some promising activity observed [27, 28]. Bevacizumab-satraplatin plus a low dose prednisolone in docetaxel-pretreated patients with metastatic castrate-
resistant prostate cancer led to a 30% decline in prostate-specific antigen, a median time to progression of 7.0 months and median overall survival of 11.2 months [29].

There is evidence that cisplatin may modulate several signal transduction pathways, including AKT, ABL, p53, MAPK/JNK/ERK [30]. Akt, also known as serine/threonine kinase or protein kinase B, pathway, has a critical regulatory role in diverse cellular processes, including cell cycle progression, protein synthesis, glucose metabolism and down-regulation of cell death [31]. Epidermal growth factor receptor (EGFR) is one of the four key molecules of this pathway, which is also important in K-Ras gene activity and in Akt kinase induction [32]. Thus, overexpression of EGFR may lead to drug resistance and potentially tumor growth [33, 34]. Additionally, there is a direct interaction between phosphatidylinositol 3 kinase (PI3K) and Ras protein for activation of PI3K/Akt cascade [35], and Ras activity is important in stimulation of PI3K/Akt signaling [36]. K-Ras-mutated tumor cells stimulate the expression of EGFR ligands, which leads to downstream signaling through phosphorylated Erk1/2 and Akt [37-39]. Moreover, NSCLC tumor cells with overexpressing Akt1 seem resistant to cisplatin [40]. Mutations in TP53 and PTEN were found in high-grade ovarian carcinoma and resistant colorectal carcinoma [41-43]. Moreover, anti EGFR therapeutics such as the monoclonal antibody cetuximab and the small molecules erlotinib, gefitinib and afatinib are registered for treatment of NSCLC and colon cancer [44-47]. Earlier, we demonstrated that post treatment with erlotinib (Tarceva®) enhanced the effect of the multitargeted antifolate pemetrexed in NSCLC cells by affecting cell cycle regulating proteins [48]. Since erlotinib is often combined with cisplatin or carboplatin, and since platinum compounds affect signaling pathways [13], we postulated that erlotinib might increase the efficacy of satraplatin. A clinical advantage might be the combination of two oral drugs. Therefore we tested whether satraplatin and erlotinib interacted synergistically in lung, colon and ovarian cancer cells, and investigated the role of DNA adduct formation, cell cycle and signaling pathways in the interaction.

MATERIALS AND METHODS

Drugs and Chemicals

Satraplatin (JM216) and JM118 were provided by GPC Biotech (Munich, Germany), and erlotinib, oxaliplatin and cisplatin were obtained from Roche, Sanofi-Aventis and Sigma (Zwijndrecht, the Netherlands), respectively. The drugs were dissolved in ethanol or dimethyl sulfoxide (DMSO), and diluted in culture medium before use. RPMI 1640 or DMEM medium, fetal calf serum (FCS), penicillin (50 IU/ml) and streptomycin (50 g/ml) were from Gibco (Gaithersburg, MD). All other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands).

Cell Culture

Seven different cell lines were included in this study: ovarian cancer (A2780, 2008), NSCLC (A549, SW1573), and colon cancer (Lov092, WiDr), while A431 (epidermoid carcinoma) was included because of its high EGFR expression. Cells were grown in monolayers and maintained in exponential growth in either RPMI 1640 (WiDr, Lov092, 2008) or DMEM (SW1573, A549, A2780, A431) supplemented with 10% heat-inactivated FCS and 20 mM HEPES at 37°C under an atmosphere of 5% carbon dioxide. Cell doubling times varied between 22-28 hr under these conditions.

Drug Sensitivity and Combination Studies

The cell growth inhibitory effects of the drugs were determined after a 72 hr exposure by the sulforhodamine B (SRB) assay in all cell lines as previously described [49]. The combinations were designed either by simultaneous treatment with JM118/erlotinib for 72 hr, or 24 hr of preincubation with either JM118 or erlotinib followed by 48 hr exposure to the combination of erlotinib/JM118 (Table 8.1). Drug ranges varied from 0.005 to 20 μM for JM118 or from 0.01-30 μM for erlotinib. Combinations were designed based on a fixed ratio of the half maximal inhibitory concentration (IC50) values of each drug.

<table>
<thead>
<tr>
<th>Mutations</th>
<th>IC_{50} (µM)</th>
<th>Erlotinib (24h)</th>
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Sub-G1 cells:

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<th>JMI18 (48h)</th>
<th>JMI18 (72h)</th>
<th>Erlotinib (72h)</th>
<th>JMI18+erlotinib (72h)</th>
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The combinations were evaluated using the multiple-drug effect analysis according to Chou and Talalay [50]. CalcuSyn software version 2.0 was used to analyse the data (Elsevier Biosoft, Oxford, UK). Results were expressed as mutually non-exclusive combination index (CI) values for every fraction affected.
(FA), while for the final evaluation we used the averaged CI at FAs of 0.50, 0.75 and 0.90, representing relevant growth inhibition values. The classification for the effect is as follows: CI <0.70 (highly synergistic/synergistic); 0.70-0.90 (moderately synergistic); 0.90-1.10 (additive); >1.10-1.45 (antagonistic).

**Measurement of Platinum-DNA Adduct Formation**

Platinum adduct formation was measured in the A2780, A549 and SW1573 cell lines. The cells were selected based on their sensitivity to the different single or combination treatment schedules. Initially adduct formation was determined after exposure to 10 M oxaliplatin, or 5 M cisplatin, JM216, JM118.

For this purpose 5x10^5 cells were seeded in 6 wells plates and drugs were added after 24 hr. Cells were harvested after 72 hr. For the combinations, different exposure times were used: 24 hr to 5 M JM118 alone or in combination or followed by 5 M erlotinib for 24 hr. Alternatively, cells were pretreated with erlotinib or JM118 followed by JM118/erlotinib for 24 hr. DNA was isolated from the cell pellets using a QIAamp DNA isolation kit (Qiagen, Venlo, the Netherlands). DNA yield and purity were checked at 260-280 nm with NanoDrop 1000 Detector (NanoDrop Technologies, Wilmington, DE, USA) and samples were analyzed on an inductively coupled plasma mass spectrometer (ICP-MS) following overnight acid hydrolyzation as previously described [51].

**Measurement of Cell Death by Analysis of Sub-G1 Region**

To evaluate the effect of the drugs on modulation of cell cycle, the cells were treated for 24, 48 and/or 72 hr with JM118, erlotinib or their combination at their IC50 concentrations. The cells were stained by highly fluorescent dye propidium iodide (PI) solution, containing 50 g/ml PI, 0.1% (Tri-) sodium citrate, 0.1% Triton X-100 and 0.1 mg/ml RNAase A. Total DNA content were measured on a Flow Cytometer (Fluorescence-Activated Cell Sorter [FACS], BD Benelux, Erembodegem-Aalst, Belgium) and the results were analyzed with the CELLQuest software, as described earlier [48, 49, 53]. Moreover, the ability of the drugs to induce cell death was evaluated by measuring sub- G1 regions during cell cycle analysis.

**Western Blot Analysis**

In order to evaluate expression of repair enzymes (the mismatch repair enzymes MSH2 and MLH1, and the excision repair enzyme ERCC1), EGFR, the modulation of signaling pathway proteins (Akt, Erk and p38) and the expression of cell cycle regulatory proteins (CDC25c, CDK2, CHK1, CHK2 and E2F-1), western blot analysis was done, as described earlier (53, 54). Twenty μg of each protein was separated on a 10% sodium dodecyl sulfate (SDS)- polyacrylamide gel and transferred to the polyvinylidene difluoride (PVDF) membrane (Immobilon®-FL, Millipore, Billerica, MA, USA). The membrane was incubated overnight with the anti- MSH2, MLH1, ERCC1, Akt, Erk, p38, CDC25c, CDK2, CHK1, CHK2 and E2F-1 (1:10000, diluted in the blocking solution; Cell Signaling Technology, Danvers, MA, USA) and mouse anti-β-actin (1:10000; Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands). For EGFR we used 1:1000 Rabbit-anti-EGFR (Cell Signaling Technology, Danvers, MA, USA) and Donkey-anti- Rabbit at a 1:2000 dilution (DAKO Cytomation, Glostrup, Denmark). The membrane was incubated with the Horseradish Peroxidase Conjugated secondary antibody (DAKO Cytomation, Glostrup, Denmark). The bands were visualized using ECL Plus Agent (Amersham Biosciences, Piscataway, NJ, USA).

**Analysis of Kinase Activity in Peptide Array**

The Pamchip array contains 136 phosphorylation sites of 144 peptides consisting of 15 amino acids. The 13 amino-carboxylic acid (R-COOH) of each peptide corresponds to known or putative phosphorylation sites in a variety of human proteins.

The A549 cells were exposed to erlotinib and/or JM118 for 2 hr at their IC50 values, harvested, rapidly frozen in liquid nitrogen and stored at -80 °C until analysis. Before analysis cells were completely lysed on ice for 20 min using Mammalian Protein Extraction Reagent (M-PER), which contains phosphatase.
and protease inhibitors (Thermo Scientific, Rockford, IL, USA). Lysates were centrifuged for 15 min at 10000 rpm; the supernatant was collected and stored at -80 °C. 40 µl control sample mix for the kinase activity array was subsequently prepared by using reaction buffer, containing ABL buffer (Westburg, Leusden, the Netherlands), 100 µM adenosine triphosphate (ATP; Sigma-Aldrich), fluorescein-labeled antibody PY20 (Exalpha, Maynard, MA, USA), 7.5 g lysate protein and DMSO. After blocking the arrays with 2% bovine serum albumin (BSA) and subsequent loading of the sample mix onto the arrays in triplicate, incubation (at 30 °C) was started for 60 cycles utilizing a PamStation®12 instrument. Repeated fluorescent imaging of each array was performed with a 12-bit charge-coupled device (CCD) camera, monitoring fluorescence intensities in real time. Spot intensity at each time point was quantified (being corrected for the local background) by the means of Bionavigator software version 5.1 (PamGene International, ’s-Hertogenbosch, the Netherlands). Based on the resulting time-resolved curves, the initial phosphorylation rate (V-ini) was calculated using specific kinetic algorithms and appropriate statistical methods.

**Statistical Analysis**

All experiments were performed in triplicate and repeated at least twice. Data were expressed as mean values ± standard error of mean (SEM). The results were analyzed by regular two-way ANOVA followed by Dunnett's multiple comparison tests. Statistical significance was set at 95% confidence interval of difference of means and a two-tailed multiplicity adjusted P value of <0.05.

**RESULTS**

**Characteristics of the Cell Lines**

Seven cell lines differing in the expression of repair enzymes and signaling pathways (Table 8.1; Figure 8.1) were used. Lovod92 is mismatch repair deficient because of the lack of expression of DNA mismatch repair protein MutL homolog 1 (MLH1); the ovarian cancer cell lines 2008 and A2780 have a somewhat higher expression of MLH1; A431, A549 and WiDr showed a higher expression of MLH1. The other DNA mismatch repair protein MSH2 was relatively high in Lovod92 and WiDr. A549, WiDR and Lovod92 have a higher expression of excision repair cross-complementation group 1 (ERCC1) compared to the other cell lines. Epidermal growth factor receptor (EGFR) is expressed in all cell lines, at a low level in A549 and SW1573 cells, but not detectable by western blotting in A2780 and WiDr cells. However, with FACS analysis a low but detectable EGFR expression could be determined in these cell lines; in the other cell lines EGFR expression by FACS was higher (data not shown). A high expression was found in A431 cells, a positive control for EGFR overexpression.

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**Figure 8.1.** Western blot of selected repair proteins using tubulin as loading control (A) and EGFR using β-actin as loading control (B). For the analysis cells in exponential growth phase were harvested and analysed on Western Blot as described in the Materials and Methods. Analysis of the repair enzymes in A431 cells was not done on the same blot as the other cell lines. Hence an example with a similar protein loading (20 µg) and a similar expression of β-actin was chosen to be added to this figure. The blots consist of a representative example of 3 separate blots.
The ovarian cell line 2008 had a TP53 (Y126C) mutation and A2780 a PTEN (K128-R130del) mutation. The NSCLC cell lines A549 had a K-Ras (G12S) mutation, and SW1573 PIK3CA (K111E), K-Ras (G12C), and p16 (M1_157del) mutations. The colon cancer cell lines Lovo92 had a K-Ras (G13D) mutation, and the WiDR a TP53 (R273H) mutation (Table 8.1). A431 cells were included because of the overexpression of EGFR (Table 8.1, Figure 8.1)

Preincubation and Simultaneous JM118 and/or Erlotinib Increased the Sensitivity

JM118 was the most active platinum analog in all cell lines investigated, except for SW1573 and 2008 cells, which were more sensitive to oxaliplatin (Table 8.1). Despite being considered as the active metabolite, SW1573 cells were less sensitive to JM118. A2780 and Lovo92 were most sensitive to JM118, while they were also most sensitive to oxaliplatin. A549 and SW1573 cells were less sensitive to JM118, but A549 was least sensitive.

The interaction between the drugs was studied at different schedules, since earlier evidence indicated that post treatment with erlotinib might prevent repair of DNA damage or delay cell cycling [48]. Simultaneous treatment with erlotinib and JM118 only showed synergism in A431 and A549 cells, and additivity in the other cell lines. Pretreatment with erlotinib was generally similar or less effective, and even antagonistic in SW1573 cells. Only in the 2008 cells was this schedule more effective (Table 8.1, Figure 8.2). Pretreatment with JM118 followed by a combination of the two drugs was the most effective schedule, showing synergism in all cell lines, which was most pronounced in A431, A549 and 2008 cells.

JM118-Erlotinib Combination Increased Platinum-DNA Adduct Formation

The effect of platinum analogs is mediated by the formation of Platinum-DNA adducts. There was a difference in adduct formation between cisplatin, oxaliplatin, JM118 and JM216, with the highest accumulation in cells treated with JM118 (Figure 8.3a; 2-6 fold higher than that of JM216). Platinum adduct levels in cisplatin and oxaliplatin -treated cells were approximately 50% of those observed in JM216 treated cells (Figure 8.3a). The highest accumulation of platinum DNA adducts was found in A2780 cells, which were the most sensitive cells, and the lowest in A549 cells, which were the least sensitive of this panel (Figure 8.3a). Platinum adduct formation after 24 hr decreased when cells were...
postincubated with erlotinib. Even simultaneous exposure to JM118 and erlotinib for 48 hr decreased the amount of adducts compared to 24 hr exposure to JM118 alone. However, 24 hr pretreatment with erlotinib increased the platinum adduct formation of a sequential 24 hr exposure to JM118. The highest accumulation was found in cells pretreated with JM118 alone, followed by a 24 hr incubation with the drug combination. This was observed in all cell lines, with the relative effect being most pronounced in SW1573 cells.

**JM118-Erlotinib Combination Modulated Cell Cycle**

Since we demonstrated that post treatment with erlotinib enhanced drug sensitivity by affecting the cell cycle, we investigated the effect of different schedules of JM118 and erlotinib on cell cycle distribution. In general, the effects on the various cell lines were comparable, with the exception of A2780 cells. A 24 hr exposure to JM118 led to an accumulation in the S phase, which turned into a G2/M accumulation after 48 and 72 hr exposure in all cell lines apart from A2780 cells (Figure 8.4). In contrast erlotinib did not show any effect at any incubation time, except for in the A2780 cell line where cells accumulated in the G1 phase. In 2008 cells simultaneous exposure to JM118 and erlotinib showed an intermediate effect with fewer cells in S-phase as compared to monotherapy regimes. However, at preincubation with JM118 the G2/M phase accumulation was retained. On the other hand, preincubation with erlotinib decreased the G2/M phase accumulation in both 2008 and A549 cancer cells compared to the JM118 pre-incubated group. In A549 cells the effect of JM118 was more dominant and erlotinib influenced the effect of JM118 to a lesser extent.

The induction of cell death (measured as sub-G1 accumulation) by JM118 was most pronounced in the most sensitive A2780 cells and least pronounced in the less sensitive A549 cells. Erlotinib had a minor effect in all cell lines. Simultaneous exposure to JM118 and erlotinib was either additive, or resulted in a decreased level of cell kill in the most sensitive cell lines (A2780, Lovo92 and WiDr). Pretreatment with erlotinib also reduced the effect in most cell lines. However, pretreatment with JM118 followed by the
A549

2008

A2780

Lovo92

Figure 8.4. Effect on cell cycle distribution in A549 NSCLC cells, 2008 and A2780 ovarian cancer cells and Lovo92 colon cancer cells. Cell cycle (G0/G1, S, G2/M phases) distribution was evaluated by FACS after 24, 48 and 72 hr. Cells were exposed to their respective IC50 values of 72 hr exposure. 24, 48 and 72 hr denotes cell cycle distribution after these time points. Combinations were evaluated only after 72 hr, at simultaneous exposure to the drugs, at 24 hr pretreatment with JM118 followed by 48 hr exposure to the combination (24 hr JM118 => 48 hr erlotinib), or at 24 hr pretreatment with erlotinib followed by 48 hr exposure to erlotinib + JM118 (24h JM118 => 48h JM118). 1: Control, 2: Erlotinib (24 hr), 3: Erlotinib (48 hr), 4: Erlotinib (72 hr), 5: JM118 (24 hr), 6: JM118 (48 hr), 7: JM118 (72 hr), 7: JM118/erlotinib (24 hr), 9: JM118/erlotinib (48 hr), 10: JM118/erlotinib (72 hr), 11: Erlotinib (24 hr) => JM118/erlotinib (48 hr), 12: JM118 (24 hr) => JM118/erlotinib (48 hr). Values are means ± SE of three separate experiments. Effects in the other two cell lines were comparable. *, p=0.0189; **, p=0.0280; ***, p=0.0014; ****, p<0.0001 compared to control.

drug combination increased the induction of cell death in the cell lines least sensitive to JM118, but not in cell lines which were already sensitive to the single drug (Table 8.1).

JM118-Erlotinib Combination Increased Phosphorylation of CDK2 and CHK1

In order to elucidate the molecular mechanism behind the cell cycle modulation, we investigated the effect of the drugs on the expression and phosphorylation of several cell cycle proteins. Erlotinib slightly increased the phosphorylation of the G2/M regulating protein CDC25c and of CDK2 in WiDR and Lovo92 cells (Figure 8.5). JM118 increased the phosphorylation of CDK2 and CHK1 in all cell lines, including 2008, A549, WiDR and Lovo92, which was also seen in the combination. Regulation of the other cell cycle proteins was variable between the cell lines studied. After 48 hr incubation, the changes in phosphorylation were less prominent, with the possible exception of the A549 cell line, where JM118 decreased the phosphorylation of all targets. Preincubation with erlotinib increased expression of E2F-1 in all cell lines tested.
Increased Phosphorylation of Akt and Erk in JM118-Erlotinib Combination

Since the Akt pathway often acts as a survival pathway, we investigated key kinases for their expression and phosphorylation. In the A2780, 2008 and WiDr cell lines, 2 hr incubation with erlotinib increased the phosphorylation of both Akt and Erk, but only of Erk in A549 cells (Figure 8.6; Table 8.S1). JM118 showed increased phosphorylation of Akt in A549, 2008 and Lov092, and of Erk in A2780 and Lov092. The combination of JM118 and erlotinib showed an additional effect on phosphorylation of Erk in Lov092, compared to that of erlotinib alone.

In SW1573 cells 24 hr incubation with erlotinib increased the phosphorylation of Akt and Erk, and in A549 of Akt (Figure 8.6; Table 8.S1). In A549 and SW1573 cells 24 hr incubation with JM118 increased the phosphorylation of both Akt and Erk, in A2780 cells of Akt alone, and in WiDR cells of Erk alone. Phosphorylation of Erk was further affected in A549 and WiDR after 24 hr incubation with JM118 and erlotinib compared to erlotinib alone.
Increased Kinase Phosphorylation on Peptide Array

In order to investigate whether the proteins were not only phosphorylated, but also affected in their function, we used a functional phosphorylation array for A549 cells. In the PAMgene array, kinase activity can be evaluated by real-time measurement of protein phosphorylation using short peptides specific for phosphorylation sites of 136 tyrosine kinases. Phosphorylation of most of these peptides was only marginally (less than 30%) affected after treatment with IC50s of erlotinib, JM118 and the combination. Hence we focused on those phosphorylation sites where one of the drugs had an effect >30%. Erlotinib decreased the phosphorylation of PTEN, STAT3, FGF-R, ERBB4, VEGFR and EPHA1 by 40-60% (Figure 8.7), while JM118 decreased that of STAT3 by more than 99% and of ERBB5, FGF-R and EPHA1 between 60-80%. Surprisingly the combination either did not further affect the phosphorylation or completely reversed the inhibition, such as for STAT3, PTEN, FGFR and EPHA1.

Figure 8.7. Kinase phosphorylation on a peptide array, including phosphatase and tensin homolog (PTEN), signal transducer and activator of transcription 3 (STAT3), fibroblast growth factor receptor (FGF-R), v-erb-b2 avian erythroblast leukemia viral oncogene homolog 4 (ERBB4), vascular endothelial growth factor (VEGF), ephrin type-A receptor 1 (EPHA1). A549 cells were exposed for 2 hr to IC50 concentrations of erlotinib and/or JM118. Phosphorylation activity in untreated cells was set at 100% for each separate kinase. Values are means ± SE of experiments in triplicate.
DISCUSSION

The present study demonstrated that the combination of JM118 and the EGFR inhibitor erlotinib, showed a schedule dependent synergism in a panel of NSCLC, ovarian and colon cancer cell lines with different molecular properties. However, simultaneous exposure was least effective in most cell lines. The most synergistic schedule was JM118 followed by the combination of erlotinib and JM118, even in cells with a Ras mutation. Interestingly the most pronounced synergism was found in cells least sensitive to JM118, such as A549 cells, and in A431 cells, which were most sensitive to erlotinib. The synergistic effect was associated with increased platinum-adduct formation, increased cell kill and increased Akt and Erk phosphorylation.

Erlotinib is an EGFR inhibitor [45, 46] and is an effective treatment for tumors with activating EGFR mutations and no mutations in the RAS pathway [47]. In earlier studies it was found that erlotinib and gefitinib showed synergism with DNA damaging drugs when given after the drug being used in combination, such as the antifolate pemetrexed [48, 52]. However, in our experiments, efficacy of the combination was also found in cells with an intrinsically low sensitivity to erlotinib. Apparently the effect of erlotinib is sufficient to inhibit DNA repair pathways as can be concluded from the increased platinum-DNA adduct formation and cell kill. This can possibly be explained by two different effects of the combination, the effect on the cell cycle proteins and on the survival pathways as was observed earlier for oxaliplatin [53]. The accumulation of cells in the G2/M phase was associated with changes in phosphorylation of cell cycle proteins that regulate the transition from one phase to another, such as CDC25c and CHK1 and CHK2. Moreover, JM118 induced phosphorylation of Akt, which was neutralized by erlotinib treatment. Similar changes were observed for combination of EGFR inhibitors with antifolates and cisplatin [30, 48].

To the best of our knowledge, this is the first study evaluating the synergistic effect of JM118 and erlotinib. The effect of erlotinib on cell signaling proteins was moderate and varied between the cell lines studied, possibly due to their different properties. In wild-type cells, especially with activating mutations, erlotinib will decrease phosphorylation of EGFR and downstream proteins [54, 55], but in cells with mutant EGFR and Ras genes, these effects are not expected. Ono et al. [56] hypothesized that in cells with mutant EGFR, the cells may depend on the EGFR pathway for their survival, thus making them sensitive to EGFR inhibitory drugs. Conversely, cells with wild type EGFR gene may depend on several receptors [46, 57], making the EGFR pathway redundant; hence, the cells become more resistant to EGFR inhibitory drugs. [55].

In the present study, having shown an increased phosphorylation of Erk after 2 hr incubation with both drugs, the cell lines were synergistic in the combination study. Even the cell lines with either increased phosphorylation of both Akt and Erk or no modulation were additive to synergistic in the combination study. This indicates that the phosphorylation of Erk seems important in response to the combination and Akt can counteract this effect, which needs further investigation. However, with a longer exposure time, the effect of the drugs on the cell signaling proteins was more cell line de-pendent.

Consequently, it seems that preincubation with JM118 is the best schedule for the combination with erlotinib. The synergistic effect of the combination regimen resulted in an increased accumulation of platinum adducts in the DNA, and thereby enhanced the disruption of the cell signaling pathways, which led to growth inhibition. Finally, the results suggest the ability of JM118 in targeting key pathways and its synergism with erlotinib.

CONFLICT OF INTEREST
The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS
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SUPPLEMENTARY MATERIALS
Supplementary material is available on the publishers web site along with the published article.
REFERENCES


Supplementary Table

**Table 8.S1.** The effects of single and combination treatment on phosphorylation of Akt and Erk signaling pathways in different cell lines after 2 and 24 hours incubation.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gene</th>
<th>2 hours</th>
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<th>2 hours</th>
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</thead>
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<tr>
<td>2008</td>
<td>Akt</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>Erk</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>+</td>
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<td>+</td>
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<tr>
<td></td>
<td>Erk</td>
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<tr>
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<td>+</td>
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<tr>
<td></td>
<td>Erk</td>
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<td>–</td>
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<td>+</td>
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<tr>
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<td></td>
<td>Erk</td>
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<tr>
<td>Lovo92</td>
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<td>0</td>
<td>+</td>
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Note: (+): increased phosphorylation; (–): decreased phosphorylation; (0): no obvious change in phosphorylation compared to the control group.
AKT1 and SELP polymorphisms predict risk of developing cachexia in pancreatic cancer patients

Abolfazl Avan1*, Amir Avan1,2*, Tessa Y. S. Le Large1, Andrea Mambrini3, Niccola Funel4, Mina Maftouh1, Majid Ghayour-Mobarhan2, Maurizio Cantore3, Ugo Boggi4, Godefridus J. Peters1, Paola Pacetti3*, Elisa Giovannetti1,4*§

1Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands; 2Biochemistry of Nutrition Research Center, and Department of New Sciences and Technology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran; 3Department of Medical Oncology, Carrara Civic Hospital, Carrara, Italy; 4Start-Up Unit, University of Pisa, Pisa, Italy
*These authors equally contributed to the study

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AKT1 and SELP polymorphisms predict the risk of developing cachexia in pancreatic cancer patients

ABSTRACT: Pancreatic ductal adenocarcinoma (PDAC) patients have the highest risk of developing cachexia, which is a direct cause of reduced quality of life and shorter survival. Novel biomarkers to identify patients at risk of cachexia are needed and might have a substantial impact on clinical management. Here we investigated the prognostic value and association of SELP-rs6136, IL6-rs1800796 and AKT1-rs1130233 polymorphisms with cachexia in PDAC. Genotyping was performed in DNA from blood samples of a test and validation cohorts of 151 and 152 chemo-naive locally-advanced/metastatic PDAC patients, respectively. The association of SELP-rs6136, IL6-rs1800796 and AKT1-rs1130233 polymorphisms with cachexia as well as the correlation between cachexia and the candidate polymorphisms and overall survival were analyzed. Akt expression and phosphorylation in muscle biopsies were evaluated by specific ELISA assays. SELP-rs6136-AA and AKT1-rs1130233-AA/GA genotypes were associated with increased risk of developing cachexia in both cohorts (SELП: p = 0.011 and p = 0.045; AKT1: p = 0.004 and p = 0.019 for the first and second cohorts, respectively), while patients carrying AKT1-rs1130233-GG survived significantly longer (p = 0.002 and p = 0.004 for the first and second cohorts, respectively). In the multivariate analysis AKT1-rs1130233-AA/GA genotypes were significant predictors for shorter survival, with an increased risk of death of 1.7 (p = 0.002) and 1.6 (p = 0.004), in the first and second cohorts, respectively. This might be explained by the reduced phosphorylation of Akt in muscle biopsies from patients harboring AKT1-rs1130233-AA/GA (p = 0.003), favoring apoptosis induction. In conclusion, SELP and AKT1 polymorphisms may play a role in the risk of cachexia and death in PDAC patients, and should be further evaluated in larger prospective studies. PLoS One 2014;19;9(9):e108057

Key words: cachexia; pancreatic cancer; polymorphisms, SELP, AKT1

Introduction

Cachexia is a multi-factorial, systemic syndrome characterized by pathological wasting of skeletal muscle and adipose tissue mass that leads to pronounced weight loss. It can occur in the course of several chronic illnesses, but it is most frequently observed concomitantly with malignancies [1]. In particular, patients with pancreatic ductal adenocarcinoma (PDAC) have the highest prevalence of cachexia, and often experience the most severe symptoms of this disease [2]. Recent studies showed that existing preoperative cachexia reduces the post-operative outcome of PDAC patients [3,4]. In advanced PDAC the presence of cachexia is also associated with a worse prognosis, and stabilizing weight can be crucial to prolong survival [5]. Moreover, cachexia decreases the tolerance to systemic treatments and dramatically affects the quality of life [6].

Extensive preclinical and clinical studies have evaluated the underlying mechanisms of cachexia in PDAC, revealing a complex of multiple interdependent patient- and cancer-specific components. Several hormones and tumor-derived factors can contribute to tissue catabolism and appetite regulation, leading to anorexia and fatigue, whereas symptoms such as chronic pain, nausea and pancreatic insufficiency, as well as adverse effects of anticancer therapies, might further reduce appetite and nutritional intake [7].

However, in contrast to cachectic patients with chronic pancreatitis, PDAC patients with cachexia have significantly reduced hemoglobin and albumin levels, associated to the systemic reaction to both the tumor and the inflammatory processes [4]. Specific pro-inflammatory cytokines such as interleukin 1-beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα), and interferon gamma have been shown to be associated with progressive weight loss, through the acute phase protein response in the liver [8–10]. This systemic inflammation can also convey its action via local inflammatory signaling, activated by the nuclear transcription factor kB (NF-kB), and in both mouse models and muscle biopsy specimens NF-kB–mediated Pax7 dysregulation emerged among the causes of muscle wasting in PDAC [11]. Furthermore, in cachexia the balance between the anabolism and the catabolism of proteins is
tipped toward a catabolic state resulting from activated ubiquitin proteasome and autophagy systems that promote protein breakdown, as well as from reduced Akt activity, that decreases protein synthesis [1,12]. The regulation of muscle differentiation is indeed dependent on the activation of signal transduction cascades with the complex involvement of key kinases, such as the serine/threonine kinase Akt, and previous studies demonstrated that Akt is essential to promote protein synthesis and cell survival and to block protein degradation [13]. All this evidence supports its biological relevance in the context of cachexia.

Although the variable production of the pro-inflammatory cytokines, NF-κB activation and Akt phosphorylation status depends on a variety of clinical and pathological factors, there is growing evidence that genetic variations might also affect these key determinants of cachexia. In particular, the C-allele of the IL6-rs1800796 polymorphism was associated with decreased survival and increased susceptibility to cachexia in Chinese PDAC patients [14]. Moreover, in a systematic study on genetic determinants of cachexia, out of 92 potential candidate genes with 184 polymorphisms relating to cancer cachexia, 42 polymorphisms across 33 genes have been identified for having a functional or clinical relevance in more than one study [15]. Of these 42 polymorphisms, 13 had more than one effect on clinical features associated with cancer cachexia (i.e. inflammation, loss of fat mass and/or lean mass, and reduced survival). Further pathway analysis of these candidates revealed 4 genes (ADIPOQ, IL6, NFKB1 and TLR4) interlinking two putative major networks involved in the development of cancer cachexia. However, in a recent study using these selected polymorphisms only the C-allele of the single nucleotide polymorphism (SNP) rs6136-SELP was found to be associated with weight loss of >10%, both in the first study cohort of 775, including 114 PDAC patients, and in the validation cohort of 101 cancer patients, including 6 PDAC patients [16]. The SELP gene encodes a 140 kDa protein stored in the alpha-granules of platelets and Weibel-Palade bodies of endothelial cells, which redistributes to the plasma membrane during platelet activation and degranulation and functions as a cell adhesion molecule (CAM) mediating the interaction of activated endothelial cells or platelets with leukocytes during the initial steps in inflammation [17].

However, the largest study on cancer cachectic patients (N=1797), including 35 PDAC patients, failed to validate the predictive role of 9 candidate SNPs [18]. One of the possible explanations for not being able to confirm genetic associations may be the gene–environment relations specific for the studied disease. Previous analysis supported the role of the IL6-rs1800796 and SELP-rs6136 SNPs as susceptibility biomarkers for PDAC cachexia [13,15], while our recent studies revealed a key functional role of the AKT1-rs1130233 SNP [19]. Therefore in the present study we evaluated the association of these candidate polymorphisms in the AKT1, IL6, and SELP genes with cachexia in a total population of 303 PDAC patients, in two independent cohorts.

Materials and Methods

Cachexia phenotype definition

To ensure that patients with or without cachexia were clearly distinguished, we used the “2011 International Consensus,” according to which cachexia is defined by unintended weight loss of more than 5% of body weight or weight loss of more than 2% in already depleted individuals with a body mass index (BMI) less than 20 kg/m2 over 6 months [20].

Patients

In the current study we selected a first cohort of 151 patients, enrolled between 31st March 2004 and 10th January 2009, and a second/validation cohort of 152 patients, enrolled between 15th January 2009 and 15th January 2013. All the eligible patients were chemo-naive patients with diagnosis of histologically confirmed locally advanced or metastatic PDAC, treated at the Carrara Civic-Hospital (Carrara, Italy).
Ethics

All the patients gave their written informed consent to the sample collection and analysis, and the study has received approval from the Ethics Committee of Carrara Civic-Hospital (Carrara, Italy) and Ethics Committee of Pisa University Hospital (Pisa, Italy) as a follow-up study of the research protocol entitled “Pharmacogenetics of gemcitabine-related genes in pancreas cancer: correlation with clinical outcome and tolerability”. The responsible investigators ensure that this study was conducted according to the Declaration of Helsinki, the European Guidelines on Good Clinical Practice, and relevant national and regional authority requirements.

Genotyping

Genomic DNA was extracted from blood samples at the Laboratory Medical Oncology (VUmc, Amsterdam, The Netherlands) using the QIAamp DNA Mini-Kit according to the manufacturer protocol (Qiagen, San Diego, CA). The concentration and purity of DNAs was determined with the NanoDrop-1000-Detector (NanoDrop-Technologies, Wilmington, USA). Genotype analysis of rs1800796, rs6136, and rs1130233 polymorphisms was performed using Taqman-based PCR reactions carried out in 12.5 ml total volume, using 20 ng of DNA diluted in TaqMan Universal Master Mix with specific primers and probes (SNP Genotyping Assays products C__11326893_10, C__11975277_20, and C__7489835_10, respectively). The ABI PRISM-7500 instrument (Applied Biosystems, Life Technologies, Foster City, CA) equipped with the SDS version-2.0 software was employed to evaluate the allelic content of each sample in the plate by reading the generated fluorescence.

Analysis of Akt expression and phosphorylation in homogenized muscle biopsies

Previous studies evaluated the relationships of AKT1 polymorphisms with AKT1 mRNA and protein expression in lymphoblastoids and lung cancer cells [19,21], but no data are available on the correlation between AKT1-rs1130233 and Akt1 protein expression and phosphorylation status in skeletal muscle. Therefore, we collected rectus abdominis muscle biopsies obtained during resection of primary PDAC. Small tissue pieces (about 100 mg) were immediately frozen in liquid nitrogen, using isopentane, after surgery at the University Hospital of Pisa (Pisa, Italy), according to a protocol approved by the local Hospital Ethic Committee. A total of 18 samples from chemonaive patients (nine for the AKT1-rs1130233-AA/GA and nine for the AKT1-rs1130233-GG genotype, as determined in preliminary genotyping analysis from blood samples, respectively) were evaluated.

The frozen tissues were homogenized using the micro-dismembrator, as described previously [22]. The frozen powder was extracted with 2.5% w/v sulfosalicylic acid and centrifuged (10 min, 8000 RPM) and the protein concentration in the extracts was determined using the Biorad protein assay (Life Science, Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). Total Akt1 and Akt1 phosphorylation at serine-473 (Akt [pS473]) were evaluated with specific ELISA assays (Invitrogen, Life Technologies), and normalized to standard curves of Human-total-Akt1 and Human-phospho-Akt1, as well as to protein content, as described previously [23].

Statistics

Demographic and clinical information were compared across genotype using Pearson's x² and logistic regression. In agreement with previous studies, the correlation with candidate genotypes was performed combining the less common homozygous and heterozygous genotypes [24].

To evaluate whether cachexia and the other clinical variables as well as the candidate polymorphisms affected clinical outcome, overall survival (OS) curves were analyzed from the day of treatment start to the end point (death or censoring) according to Kaplan–Meier method, and compared by log-rank. The significant prognostic variables in the univariate analysis were included in multivariate analysis, using Cox’s proportional hazards model. This analysis included a step down procedure based on the
likelihood ratio test, where hazard ratio (HR) was calculated to estimate the magnitude and the direction of the effect. Appropriate adjustment for false-positive report probability in the analysis of the polymorphisms was performed according to the Wacholder method [25].

All the analyses of the samples were done in a blinded fashion relative to clinical outcome. Data were analyzed using SPSS-20 software (IBM, IL, USA). All the analyses were two-sided and statistical significance was set at p-value of <0.05.

Results

Clinical characteristics and outcome

Patient baseline characteristics and their association with clinical outcome are summarized in Table 9.1. OS data were available from all patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>First cohort</th>
<th>OS median mo. (95% CI)</th>
<th>p-value*</th>
<th>Second/validation cohort</th>
<th>OS median mo. (95% CI)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. patients</td>
<td>151</td>
<td>12.5 (10.9–14.1)</td>
<td></td>
<td>152</td>
<td>12.0 (9.9–14.1)</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥65</td>
<td>92 (60.9%)</td>
<td>13.5 (11.7–15.3)</td>
<td>0.031</td>
<td>100 (65.8%)</td>
<td>11.6 (9.8–13.4)</td>
<td>0.864</td>
</tr>
<tr>
<td>&gt;65</td>
<td>59 (39.1%)</td>
<td>10.9 (8.4–13.4)</td>
<td></td>
<td>52 (34.2%)</td>
<td>13.3 (11.4–15.4)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>98 (64.9%)</td>
<td>12.3 (10.2–14.3)</td>
<td>0.697</td>
<td>92 (60.5%)</td>
<td>11.8 (8.2–13.8)</td>
<td>0.044</td>
</tr>
<tr>
<td>Female</td>
<td>53 (35.1%)</td>
<td>13.3 (9.5–17.1)</td>
<td></td>
<td>60 (39.5%)</td>
<td>13.7 (10.9–16.5)</td>
<td></td>
</tr>
<tr>
<td>Cachexia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yes</td>
<td>53 (35.1%)</td>
<td>9.9 (8.4–11.4)</td>
<td>0.0006</td>
<td>59 (38.8%)</td>
<td>9.1 (6.9–11.2)</td>
<td>0.005</td>
</tr>
<tr>
<td>no</td>
<td>98 (64.9%)</td>
<td>14.3 (12.4–16.2)</td>
<td></td>
<td>93 (61.2%)</td>
<td>14.2 (12.7–15.7)</td>
<td></td>
</tr>
</tbody>
</table>

*p-values were calculated with Log-rank test.
OS: Overall survival; mo, months.
doi:10.1371/journal.pone.0108057.t001

In the first cohort there were 147 deaths (event rate of 97.4%), while four patients were alive without progression while in the validation cohort, there were 144 deaths (event rate of 94.7%) and eight patients were alive without progression at last contact (January 2014), with a median follow-up for living patients of 31.4 months (range, 28.4–32.7). Median OS in the first and second cohorts were 12.5 months (95% CI: 10.9–14.1) and 12.0 months (95% CI: 9.9–14.1), respectively.

In the first and second cohorts of patients OS was significantly shorter among patients with an age of over 65 years and male gender (p = 0.031 and p = 0.044), respectively, whereas other patient characteristics were not associated with outcome.

Patients in the validation cohort had a comparable distribution of weight loss, and baseline demographic characteristics were also quite similar between the two cohorts. Importantly, cachexia was significantly associated with shorter OS in both cohorts (p = 0.00006 and p = 0.005, respectively). The first cohort included 53 cachectic patients (35.1%), with a median OS of 9.9 months (95% CI: 8.4–11.4), compared to 14.3 months (95% CI: 12.4–16.2) of non-cachectic patients, while the validation cohort included 59 cachectic patients (38.8%), with a median OS of 9.1 months (95% CI: 6.9–11.2), compared to 14.2 months (95% CI: 12.7–15.7) of non-cachectic patients (Figures 9.1A–B).
Polymorphisms and cachexia

To investigate whether there is an association between cachexia and IL6-rs1800796, AKT1-rs1130233 and SELP-rs6136 polymorphisms, we performed genotyping using genomic DNA extracted from peripheral blood samples.

Previous studies showed no differences in polymorphisms analyzed in tumors and normal tissues [26,27]. However, since high level gene amplification in tumor cells might result in homozygous genotypes in individuals who are heterozygous in the germline, we performed preliminary studies of AKT1 polymorphisms in 45 paired samples of germ-line and cancer DNA, showing identical interindividual genotypes between normal and malignant tissues [19]. Therefore, for the patients enrolled in this study the genotyping was performed in DNA extracted from the available blood samples. Genotyping was successfully carried out in all the DNA samples, and no discrepancies were found in the samples analyzed in duplicate (approximately 10%).

In the first cohort of patients the wild-type SELP-rs6136 genotype (AA) had a frequency of 46.4%, whereas the AC and CC genotypes were found in 43.7% and 9.9% of the patients, respectively. For the IL6-rs1800796 polymorphism, the frequencies of the GG, GC and CC genotypes were 59.3%, 37.4% and 3.3%, respectively. Regarding the AKT1-rs1130233 polymorphism, the GG, GA and AA variants were observed in 52.8%, 39.3% and 7.9% of the cases, respectively. Similar results were observed in the
At univariate analysis, the AKT1-rs1130233 polymorphism was associated with significantly differential OS. In particular, significantly longer survival was observed in patients harboring the GG genotype (14.3 months; 95% CI: 12.1–16.5), in comparison with patients carrying the GA/AA genotype, who had a median OS of 10.3 months (95% CI: 8.1–12.4; \( p = 0.002 \); Figure 9.1C). Similar results were observed in the validation cohort: patients with the GA and AA genotypes had a worse prognosis (median OS 9.9 months, 95% CI: 8.6–11.1), compared to the patients carrying the GG genotype (14.3 months; 95% CI: 12.1–16.8; \( p = 0.002 \)).

### Polymorphisms and outcome

Considering that cachexia is a key determinant of cancer-related death, we hypothesized that the candidate polymorphisms associated with cachexia might also affect the clinical outcome.

Second cohort of patients, as reported in the Table 9.2. Indeed, no significant differences were measured both the baseline demographic characteristics and in the frequencies of the candidate polymorphic genotypes in the two cohorts of PDAC patients.

All the polymorphisms followed Hardy–Weinberg equilibrium, as calculated with the SNP analyzer software (http://snp.istech21.com/snpanalyser/2.0/, Table 9.S1) and their allelic frequencies were comparable with those reported in Caucasian populations, and in NCBI and NCI-SNP500 databases. No significant correlations were detected between genotype and baseline demographic characteristics (i.e. age and sex, data not shown).

By grouping patients with IL6-rs1800796-GG versus those with GC or CC genotypes we did not find an association with cachexia (Table 9.2). Conversely, a correlation was observed between the AKT1-rs1130233 genotype and cachexia, with significantly higher proportion of patients harboring at least one A-allele having cachexia (i.e. 37 out of 81, versus 16 out of 70 GG patients, \( p = 0.004 \)). As shown in Table 9.2, the SELP-rs6136 polymorphism was also significantly \((p = 0.011)\) associated with cachexia. In particular, 44% of the patients harboring the SELP-rs6136-AA variants experienced cachexia, compared to 24% of the patients harboring the SELP-rs6136-AC/CC genotype.

Remarkably, we observed similar results in the validation cohort of patients, showing that individuals who carry the AKT1-rs1130233-GG genotype or the C-allele of the SELP-rs6136 polymorphism were at reduced risk of developing cachexia (Table 9.2). These results were confirmed by unconditional logistic regression, which calculated ORs and their 95% CI for association with cachexia phenotype of each individual SNP, as reported in Table 9.S2.

### Table 9.2. Correlation between cachexia and candidate SELP, AKT1 and IL-6 SNPs.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Cachexia</th>
<th>Genotype</th>
<th>First cohort</th>
<th>Second cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Patients %</td>
<td>n (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( p )-value*</td>
<td>n (%)</td>
</tr>
<tr>
<td>SELP-rs6136</td>
<td>Yes</td>
<td>AA</td>
<td>36 (23.8)</td>
<td>36 (23.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AC/CC</td>
<td>17 (11.2)</td>
<td>23 (15.1)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>AA</td>
<td>45 (29.8)</td>
<td>40 (26.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AC/CC</td>
<td>53 (35.2)</td>
<td>53 (34.8)</td>
</tr>
<tr>
<td>AKT1-rs1130233</td>
<td>Yes</td>
<td>GG</td>
<td>16 (10.1)</td>
<td>21 (13.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GA/AA</td>
<td>37 (24.5)</td>
<td>36 (23.3)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>GG</td>
<td>54 (35.7)</td>
<td>53 (34.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GA/AA</td>
<td>44 (29.7)</td>
<td>40 (27.6)</td>
</tr>
<tr>
<td>IL6-rs1800796</td>
<td>Yes</td>
<td>GG</td>
<td>17 (11.2)</td>
<td>18 (11.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GC/CC</td>
<td>36 (23.4)</td>
<td>41 (27%)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>GG</td>
<td>43 (28.5)</td>
<td>34 (22.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GC/CC</td>
<td>55 (36.9)</td>
<td>59 (38.8)</td>
</tr>
</tbody>
</table>

\(^{*}\)p-values were calculated with Fisher’s exact test.

SNPs, single nucleotide polymorphisms.

Note: SELP and IL6 SNPs was detectable in all the samples, while the AKT1 genotype could not be determined in 2 of the patients of the second cohort.

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months, 95% CI: 8.6–11.1), compared to the patients harbouring the GG genotype (14.3 months, 95% CI: 11.7–16.8; \( p = 0.004 \); Table 9.3; Figure 9.1D). To keep at minimum the probability to find a statistically significant difference purely by chance, the usual nominal level (\( p = 0.05 \)) has been lowered to 0.017 by Bonferroni adjustment for multiple comparisons. After the Bonferroni adjustment, the AKT1-rs1130233 SNP was still significantly correlated to OS. In contrast, SELP-rs61316 and IL6-rs1800796 polymorphisms were not associated with the outcome, as reported in Table 9.3.

In the multivariate analysis, cachexia and age >65 were significantly associated with increased risk of death (Table 9.4). The Cox proportional hazard regression model also showed the prognostic significance of the AKT1-rs1130233 polymorphism. In particular, the GA/AA genotype emerged as a significant predictor for shorter survival, with an increased risk of death of 1.7 (95% CI: 1.2–2.4; \( p = 0.002 \)) and 1.6 (95% CI: 1.2–2.3; \( p = 0.004 \)), in the first and second cohorts, respectively.

**AKT1-rs1130233 and expression of Akt1 and phospho-Akt1 in skeletal muscle**

The significant association of the AKT1-rs1130233 polymorphism with cachexia and clinical outcome prompted us to perform an exploratory study on its role on the level and activity of Akt1 in a panel of muscle biopsies. As illustrated in Figure 2, the samples with the GA or AA genotypes had a trend toward a significantly lower protein expression (approximately 230%) of Akt1 in comparison to the samples with AKT1-rs1130233-GG genotype (\( p = 0.050 \)). These results were similar to the reduction in phospho-Akt1 levels, resulting in a significant lower average level of phosphorylation at Ser437 of Akt1 in the GA/AA samples compared to the GG samples (\( p = 0.006 \)). However, when we normalized the phospho-Akt1 levels to the total-Akt1 levels in each sample we observed that in all except one case, the samples with GA/AA genotype had a lower ratio between phospho-Akt1 and total Akt1 vs. the samples with the GG genotype (Figure 9.S1, \( p = 0.003 \)). These data suggest that the AKT1-rs1130233-GA/AA genotypes might confer a reduced activity to Akt1, and thus reduce the antiapoptotic activity of this pivotal regulator of apoptotic signaling.

### Table 9.3. Clinical outcome according to candidate SELP, AKT1 and IL-6 SNPs.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>First cohort</th>
<th>Second/validation cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OS median mo. (95% CI)</td>
<td>( p )-value</td>
</tr>
<tr>
<td>SELP rs61316</td>
<td>AA</td>
<td>11.4 (8.4–14.4)</td>
<td>0.665</td>
</tr>
<tr>
<td></td>
<td>AC/CC</td>
<td>12.7 (10.1–15.2)</td>
<td>0.002</td>
</tr>
<tr>
<td>AKT1 rs1130233</td>
<td>GG</td>
<td>14.3 (12.1–16.5)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>GA/AA</td>
<td>10.3 (8.1–12.4)</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-6 rs1800796</td>
<td>GG</td>
<td>11.5 (7.1–16.0)</td>
<td>0.259</td>
</tr>
<tr>
<td></td>
<td>GC/CC</td>
<td>12.5 (10.8–14.3)</td>
<td>0.019</td>
</tr>
</tbody>
</table>

OS: overall survival; mo: months.
doi:10.1371/journal.pone.0108057.t003

### Table 9.4. Factors associated with overall survival in the multivariate analysis.

<table>
<thead>
<tr>
<th>Covariates for OS</th>
<th>First cohort</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>df</td>
<td>( p )-value</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( &lt;65 )</td>
<td>1 (ref)</td>
<td>1</td>
<td>0.032</td>
</tr>
<tr>
<td>( &gt;65 )</td>
<td>1.4 (1.0–2.0)</td>
<td>1</td>
<td>1.0 (0.7–1.4)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.1 (0.8–1.5)</td>
<td>1</td>
<td>1.3 (1.0–1.9)</td>
</tr>
<tr>
<td>Female</td>
<td>1 (ref)</td>
<td>1</td>
<td>0.046</td>
</tr>
<tr>
<td>Cachexia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2.2 (1.6–3.2)</td>
<td>1</td>
<td>1.0 (1.2–2.3)</td>
</tr>
<tr>
<td>No</td>
<td>1 (ref)</td>
<td>1</td>
<td>0.006</td>
</tr>
<tr>
<td>AKT1 rs1130233</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>1 (ref)</td>
<td>1</td>
<td>0.002</td>
</tr>
<tr>
<td>GA/AA</td>
<td>1.7 (1.2–2.4)</td>
<td>1</td>
<td>1.6 (1.2–2.3)</td>
</tr>
</tbody>
</table>

\( df \): degrees of freedom; HR: hazard ratio; OS: overall survival.
doi:10.1371/journal.pone.0108057.t004
Discussion

In the present study we demonstrated the importance of AKT1-rs1130233 polymorphism as a predictive marker of risk of cachexia and death in patients with locally-advanced or metastatic PDAC. Our pharmacogenetic analysis also identified an association between cachexia and the SELP-rs6136-AA genotype, in agreement with a previous study in 120 PDAC patients [16]. Conversely, no statistically significant correlation was detected for the G-allele of the rs1800796 polymorphism in the IL6 gene, which was associated with increased susceptibility to cachexia and decreased survival time of stage II and III Chinese PDAC patients [14]. These discrepancies could suggest that pharmacogenetic associations are not always reproducible in studies in populations with different ethnic backgrounds, as well as in different clinical stages. However, the minor allele frequencies, of approximately 4%, in our cohorts, greatly limited the statistical power for the analysis of this polymorphism. Larger studies with homogeneous settings of patients are essential to investigate the role of emerging biomarkers before planning of prospective trials.

PDAC patients have the highest risk of developing cachexia among the gastrointestinal tumors, and it has been shown that cachexia is correlated with poor prognosis, reduced treatment tolerance and a significant reduction in the quality of life of these patients [28,29].

Cachexia can be caused by a complex interplay of mechanisms including medical problems such as diabetes, tumor stage, and duodenal or common bile duct obstruction, which cause pain, nausea, dysphagia, gastroparesis, pancreatic insufficiency and malabsorption [30]. Recent studies have shown that also neural invasion, which commonly occurs in PDAC, is related to cachexia. Nerve damage from PDAC can indeed activate astrocytes, which subsequently induce lipolysis and muscle atrophy [31]. Furthermore, the increase in the sympathetic nervous system activity might cause lipolysis in adipose tissue and muscle atrophy [32]. In addition to these mechanical factors, several other mechanisms have been proposed to drive the pathophysiology of PDAC cachexia and there is evidence that anorexia and hypercatabolism can be triggered by cytokines, circulating hormones, neuropeptides, neurotransmitters, and tumor-derived factors.

Several studies showed that increased levels of cytokines, such as IL-6, were associated with weight loss and poor prognosis in PDAC patients [8–10]. However, the variable predisposition to cachexia may be also due to the patient’s genotype, and a comprehensive pharmacogenetic study demonstrated the association of cachexia with the rs6136 polymorphism of the gene SELP. This gene encodes the cell adhesion protein P-selectin, which was found to be upregulated in murine and rats models of cachexia caused by both acute and chronic inflammatory insults [16]. These data revealed that P-selectin has a
relevant role in both animal models and in cachectic cancer patients. However, no data are yet available on its role as a risk factor or as a potential mediator of the cachectic process.

Since apoptosis is reported to take place in wasting muscle in cachexia, several other studies evaluated the key role of Akt1 in developing cancer cachexia [33–35]. Akt1 is a serine/threonine kinase acting as a critical mediator of growth factor-induced survival. Survival factors can suppress apoptosis in a transcription- independent manner by activating Akt1, which then phosphorylates and inactivates components of the apoptotic machinery [32,36]. Moreover, in skeletal muscle, Akt1 plays a very central role in the control of both muscle protein synthesis, via mTOR, and protein degradation, via the transcription factors of the FoxO family. This suggests a pivotal role in excessive loss of muscle mass associated with several diseases, including myopathies and muscular dystrophies, as well as in cachexia associated with systemic disorders such as cancer, diabetes, sepsis and heart failure [13]. Schmitt and colleagues demonstrated a cachexia-associated loss of Akt-dependent signaling in human skeletal muscle of cachectic patients compared to non-cachectic patients, using muscle biopsies from 16 PDAC patients undergoing pancreatectomy [33]. Notably, AKT1 is a highly polymorphic gene, and functional SNPs might affect Akt1 levels and influence apoptosis induction [21].

Therefore, in the current study we investigated the association with cachexia and the prognostic value of the candidate functional polymorphisms IL6-rs1800796, AKT1-rs1130233 and SELP-rs6136 in a first cohort of 151 patients with locally-advanced or metastatic PDAC. Then we validated the results by replicating the association study in an independently recruited group of 152 patients.

To the best of our knowledge this is the first study demonstrating that individuals who carried the A-allele for AKT1-rs1130233 polymorphism were at increased risk of developing cachexia. Moreover, these patients survived significantly shorter, compared to patients carrying the GG genotype. Remarkably, the Cox proportional hazards regression model used for the multivariate analysis illustrated the independent prognostic value of AKT1-rs1130233.

Of note, the AKT1-rs1130233 is a synonymous polymorphism, i.e. a polymorphism where the change in the base in the DNA sequence does not alter the amino acid encoded due to the redundancy of the genetic code. Because synonymous SNPs do not change the composition of the protein product, they have largely been assumed to exert no discernible effect on gene function or phenotype. However, studies on artificial site-directed silent mutagenesis of synonymous codons in several genes support the hypothesis that altered translation kinetics of mRNA, caused by altered translation kinetics and folding, might affect final protein conformation [37]. Moreover, Kimchi-Sarfaty and collaborators demonstrated that the P-glycoprotein inhibitors cyclosporin and verapamil were less effective against proteins that were produced from polymorphic haplotypes that did not change the amino acid sequence but slow down the ribosome traffic at the corresponding mRNA regions [38]. These alterations may thus affect the cotranslational folding pathway, resulting in a different final conformation and function. Although we could not test this hypothesis because of the lack of conformation-sensitive mono- clonal antibodies for Akt1, previous studies in different tissue types showed that the AKT1-rs1130233-AA variant correlated with lower AKT1 mRNA expression [19], and with lower protein levels, contributing to lower apoptotic response [21].

To gain further insight into the mechanisms behind our findings, we performed additional studies showing a significant association of Akt1 phosphorylation status in muscle biopsies and the AKT1-rs1130233 polymorphism. In particular we observed a significant reduction of the phosphorylation at Ser437 of Akt1 in the GA/AA samples compared to the GG samples. These results suggest that the AKT1-rs1130233-GA/AA genotypes might reduce the activity to Akt1 and favour the induction of apoptosis, which in turn causes muscle atrophy and increases cachexia, in agreement with the clinical results.

Remarkably, a recent study by He and colleagues showed that tumor-secreted microvesicles contain an elevated expression of microRNA-21 (miR-21) and induce myoblast apoptosis in cancer cachexia via a Toll-like receptor 7-c-Jun N-terminal kinase- dependent pathway [39]. We have recently shown that
miR-21 is up-regulated and acts as an oncogene in pancreatic intraductal papillary mucinous neoplasms and PDAC [40,41]. Moreover, we demonstrated that modulation of Akt1 phosphorylation and apoptosis induction may contribute to the prognostic role of miR-21, as well as in gemcitabine chemoresistance [42]. Indeed, among the multiple targets of miR-21 in PDAC we showed the key role of PTEN, leading to AKT1 regulation. Therefore, we might hypothesize that the different AKT1 genotypes might affect its inhibition by PTEN after stimulation by miR-21, favouring tumor-induced muscle wasting through apoptosis induction.

However, previous studies reported controversial relationship between miR-21 expression and PTEN regulation, both in the preclinical and clinical setting [43], as well as on the functional role of candidate AKT1 SNPs [44–46]. More genotype-phenotype correlation studies and functional analyses of other critical genes involved in the Akt pathway are warranted. Further research to elucidate the intricate mechanisms involved in the induction and maintenance of PDAC cachexia, should aid in the development of future therapeutic targets. In particular, it remains to be determined if modulation of phosho-Akt by specific drugs might alter the development of cachexia. Akt1 might indeed be a candidate therapeutic target in cancer cachexia and even survival of PDAC, after the selection of the patients according to their genotype.

A major strength of the present study is that it was carried out in a homogeneous setting of patients with pancreatic cancer. The results of multivariate analysis indicate the noteworthiness of the prognostic role of AKT1-rs1130233. Moreover, the minor allele frequency of this polymorphism in a random Caucasian population is frequent (i.e., 28% according to the SNP-NCBI cancer database). Thus, these findings might be relevant to a large number of patients. Conversely, the main limitations of this study include the retrospective explorative study design and the lack of prospective randomized studies on the potential predictive role of AKT1-rs1130233 for chemotherapy activity.

Conclusion and Future Perspective

AKT1-rs1130233 and SELP-rs6136 polymorphisms emerged as a predictive risk factor of developing cachexia in locally-advanced and metastatic PDAC. Moreover AKT1 polymorphisms may play a prognostic role. Since pancreatic cancer is such a lethal disease, any biomarker that can help to better stratify patients for developing cachexia might have crucial clinical applications. Ultimately, validation of the value of the emerging candidate polymorphisms in future prospective trials will offer new tools to improve the clinical management of advanced PDAC patients.

Supporting Information

Figure 9.S1 Phospho/Total Akt1 expression in muscle samples according to the AKT1-rs1130233 polymorphism. Bar graphs illustrating the mean±SD expression of the ratio of total Akt1 and phospho-Akt1 in muscle samples from patients with differential AKT1-rs1130233 genotypes. *p<0.05. (PPT)

Table 9.S1 Genotyping of PDAC patients for the candidate SELP, AKT1 and IL-6 SNPs. (DOC)

Table 9.S2 Logistic regression analysis of cachexia and SELP, AKT1 and IL-6 SNPs. (DOC)

Author Contributions

Conceived and designed the experiments: EG PP MGM GJP. Performed the experiments: Abolfazl Avan Amir Avan MM NF TYS. Analyzed the data: Abolfazl Avan Amir Avan MM NF TYSL EG PP MGM GJP. Contributed reagents/materials/analysis tools: PP NF AM MC UB. Contributed to the writing of the manuscript: Abolfazl Avan Amir Avan PP TYSL EG.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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Correspondence to: Elisa Giovannetti, MD, PhD, Department of Medical Oncology, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, the Netherlands. E-mail: e.giovannetti@vumc.nl

References


### Table 9.S1. Genotyping of PDAC patients for the candidate SELP, AKT1 and IL-6 SNPs

<table>
<thead>
<tr>
<th>SNP</th>
<th>First cohort</th>
<th>Second/validation cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients n (%)</td>
<td>HWE p-value</td>
</tr>
<tr>
<td>SELP-rs6136</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>81 (53.6)</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>60 (39.7)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>10 (6.6)</td>
</tr>
<tr>
<td>AKT1-rs1130233</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>70 (46.4)</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>65 (43.1)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>16 (10.6)</td>
</tr>
<tr>
<td>IL6-rs1800796</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>91 (60.3)</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>51 (33.8)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>9 (6.0)</td>
</tr>
</tbody>
</table>

HWE: Hardy–Weinberg equilibrium

### Table 9.S2. Logistic regression analysis of cachexia and SELP, AKT1 and IL-6 SNPs

<table>
<thead>
<tr>
<th>SNP</th>
<th>Risk genotype</th>
<th>df</th>
<th>First cohort</th>
<th>Second cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>OR (95%CI)</td>
<td>p-value</td>
</tr>
<tr>
<td>SELP-rs6136</td>
<td>AA</td>
<td>1</td>
<td>2.4 (1.1-5.0)</td>
<td>0.017</td>
</tr>
<tr>
<td>AKT1-rs1130233</td>
<td>GA-GA</td>
<td>1</td>
<td>3.0 (1.4-6.3)</td>
<td>0.003</td>
</tr>
<tr>
<td>IL6-rs1800796</td>
<td>GG</td>
<td>1</td>
<td>0.6 (0.3-1.2)</td>
<td>0.139</td>
</tr>
</tbody>
</table>

df: degree of freedom; OR: odds ratio; SNPs: single nucleotide polymorphisms
CHAPTER 10

SNPs in PI3K-PTEN-mTOR and Brain Metastases in NSCLC—Letter

Abolfazl Avan*,¹ Mina Maftouh*,¹ Amir Avan,¹,² Carmelo Tibaldi,³ Paolo A. Zucali,⁴ Elisa Giovannetti¹#

¹Department of Medical Oncology, VU University Medical Center, Amsterdam, the Netherlands; ²Department of New Sciences and Technology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran; ³Department of Oncology, Azienda USL-6 of Livorno, Livorno, Italy; ⁴Department of Medical Oncology and Hematology, Istituto Clinico Humanitas, Rozzano, Milan, Italy.

*These authors equally contributed to the study
SNPs in PI3K/PTEN/mTOR and brain metastases in NSCLC—Letter

We have read with interest the study by Li and colleagues (1) on single-nucleotide polymorphisms (SNP) predicting brain metastases in non–small cell lung cancer (NSCLC). Patients with the GT/GG genotype of AKT1-rs2498804, CT/TT of AKT1-rs2494732, and AG/AA of PIK3CA-rs2699887 had higher risk of brain metastasis. These data support the feasibility of studying germinal polymorphisms for risk stratification. However, several points should be discussed in more detail.

First, the choice to focus on NSCLC is valuable, as treatment with prophylactic cranial irradiation is a matter of debate in this tumor. However, according to the "rule of tens," in a multivariate analysis there should be a minimum of 10 events per predictor variable. As 16 SNPs were analyzed, a total of 160 patients would have to have brain metastases to prevent overfitting, because the results from an overfitted model are not generalizable to other populations. Another critical obstacle to the successful development of a genotype-based test is the high number of spurious associations (2). To avoid false-positive associations, a Bonferroni correction considering all the studied SNPs would require a P value of <0.05/16 = 0.003 for statistical significance.

Emerging SNPs need to be validated in independent cohorts, and our dataset from stage IIIB/IV NSCLC (3) confirmed the association of AKT1-rs2498804, but not of PIK3CA-rs2699887, with brain metastases (Table 10.1). These conflicting data might be explained by several factors, such as ethnicity, sample size, different clinical settings, histotypes, stage, and treatment. However, Li and colleagues did not describe treatment details of their cohort, while all our patients received gefitinib. Similarly, although 50% of their patients were never-smoker, no information was provided on EGFR mutations or ALK translocations. Because almost half of ALK+ patients treated with crizotinib have central nervous system relapse (4), we wonder whether the same results would have been obtained after adjustment for EGFR/ALK genetic aberrations and targeted treatments. Ultimately, the role of AKT1-rs2498804 in clinical decision-making should be validated within prospective trials in homogeneous patient cohorts.

In addition to pharmacogenetic trials, further research is needed to unravel the functional significance of these polymorphisms. Specifically, AKT1-rs2498804, located at 30 untranslated region, may affect gene expression through changes in transcription factor–binding sites, microRNA target sequences, and/or splicing variants. Although this SNP has not been tested in experimental models (5), it could be evaluated at least in silico, using publicly available software such as PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=4TF_8.3) to detect transcription factor–binding sites, MicroSNiper (http://cbdb.nimh.nih.gov/microsniper) for prediction of SNP effects on microRNA targets, and Human Splicing Finder (http://www.umd.be/HSF/) to study pre-mRNA splicing. In particular, additional studies should investigate whether differential expression levels of candidate microRNAs might explain the fact that this polymorphism was not associated with metastasis at sites other than the brain. Finally, direct evidence of the effects on protein expression could be obtained by immunohistochemistry of NSCLC primary and metastatic tissues.

In conclusion, we thank Li and colleagues for their study, but we believe that additional parameters are essential to validate candidate SNPs beyond already available clinical factors for predicting brain metastases in NSCLC.

Table 10.1. Polymorphisms and brain metastases in patients with advanced NSCLC treated with gefitinib

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Patients, N</th>
<th>Events, N (%)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT1-rs2498804</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>36</td>
<td>3 (8)</td>
<td></td>
</tr>
<tr>
<td>GT+GG</td>
<td>54</td>
<td>17 (31)</td>
<td>0.010</td>
</tr>
<tr>
<td>PIK3CA-rs2699887</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>79</td>
<td>15 (19)</td>
<td></td>
</tr>
<tr>
<td>AG+AA</td>
<td>11</td>
<td>5 (45)</td>
<td>0.062</td>
</tr>
</tbody>
</table>

NOTE: Polymorphic loci in AKT1 and PIK3CA were assessed in DNA isolated from blood samples and/or paraffin-embedded tumors from 90 patients with stage IIIB/IV NSCLC treated with gefitinib (ClinicalTrials.gov ID-NCT00831454), as described previously (3).

*P values were calculated by the Fisher exact test.

Corresponding Author:
Abolfazl Avan, M.D., Ph.D., Department of Medical Oncology, VU University Medical Center, CCA Room 1.42, De Boelelaan 1117, 1081 HV Amsterdam, the Netherlands. Phone: 31-20-4442267; Fax: 31-20-4443844; E-mail: e.giovannetti@vumc.nl

1081 HV Amsterdam, the Netherlands. Phone: 31-20-4442267; Fax: 31-20-4443844; E-mail: e.giovannetti@vumc.nl

Chapter 10

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Correspondence to: Elisa Giovannetti, MD, PhD, Department of Medical Oncology, VU University Medical Center, CCA Room 1.42, De Boelelaan 1117, 1081 HV Amsterdam, the Netherlands. Phone: 31-20-4442267; Fax: 31-20-4443844; E-mail: e.giovannetti@vumc.nl

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DISCUSSION

AND

SUMMARY
Chapter 11

Discussion
Discussion

Part 1: Chemotherapy-induced Neurotoxicity

Neurologic complications can be seen throughout the course of cancer diagnosis and treatment. Some toxicities may develop during or years after completion of treatment, which makes the diagnosis challenging. Neurologic disorders affect approximately 15% of patients with cancer and may diminish the patient’s quality of life and directly shorten survival. They may be associated with metastatic complications of the cancer in the brain, cranial nerves, spine, leptomeninges, and peripheral nerves, or nonmetastatic ones, including infection, toxic metabolites, vascular, paraneoplastic, or related to chemo-radiotherapy. Peripheral neurotoxicity is a major dose-limiting toxicity of chemotherapy and a frequent source of painful and disabling symptoms that diminish quality of life in patients with cancer. Early recognition may result in treatment and improvement of adverse neurologic symptoms as well as avoid permanent neurologic damage. It is also important to recognize neurologic complications of cancer early and to appropriately distinguish symptoms directly related to the cancer, such as metastatic involvement, from those associated with other neurologic disorders or with cancer medication (1). Moreover, certain cancers, such as small cell lung cancer, multiple myeloma, thymoma, and Waldenstrom macroglobulinemia are associated with neuropathy, which need to be considered.

Clinical manifestations of neurological side effects may vary depending upon the type, dose, and duration of treatment. Moreover, the type of the affected nerve fibers, i.e. sensory, motor, or autonomic, determines the signs and symptoms. Sensory nerve cell bodies reside in the dorsal root ganglia, unlike motor neuron cell bodies, which reside in the spinal cord and are not protected by the blood brain barrier. Hence, sensory nerve cell bodies have a much greater exposure to circulating drugs. Common sensory symptoms include symmetric painful paresthesia in the hands and feet, allodynia, loss of deep tendon reflexes, and diminished proprioception and more proximal vibratory sensitivity impairment.

The chemotherapy agents most commonly associated with peripheral neuropathy (Table 11.1) are taxanes, vinca alkaloids, platinum compounds, proteasome inhibitors (bortezomib), and antiangiogenic compounds (thalidomide). Given the clinical burden of chemotherapy-induced peripheral neuropathy, there is a growing interest in better understanding its pathophysiology, standardizing evaluation, and developing effective treatments.

Platinum compounds are widely used in various anticancer regimens. Thus, in the first part of the thesis, a systematic review of the literature is provided for pathophysiology, pathogenesis, and characteristics of platinum-induced neurotoxicity along with the best available evidence on the preventive and therapeutic strategies (2). Platinum neurotoxicity may present not only with an acute or chronic peripheral neuropathy, but also as central, e.g. chemo brain (short-term memory and concentration problems and an inability to resume high-level tasks as a result of high dose chemotherapy) and ototoxicity (sensorineural hearing loss). The incidence of peripheral neurotoxicity is variable and ranges from 30% to 40% of patients with cancer treated with chemotherapy (1), with some studies reporting an incidence as high as 90% (3).

Peripheral neurotoxicity of platinum compounds is associated with several molecular alterations, including dorsal root ganglia cytotoxic inflammatory changes, mitotoxicity and enhanced oxidative stress, voltage-gated ion (sodium/potassium/calcium) channel dysfunction, functional impairment of ion channels of the transient receptor potential family, induction of neuronal apoptosis in dorsal root ganglia, and demyelination (4). Accumulation of platinum products, mainly in the dorsal root ganglia, as well as peripheral neurons may provoke chronic neuropathy. Since these neurons are post-mitotic and not dividing, the formation of DNA adducts is not lethal, but results in DNA-strand breaks. The extent of DNA crosslinks in dorsal root ganglia and the degree of neurotoxicity is related to the total cumulative dose of platinum agent (5). Cisplatin produces more adducts in the dorsal root ganglia
compared to oxaliplatin, which may explain its higher neurotoxicity. Platinum adducts may also affect neurons, while brain and spinal cord are protected by the blood brain barrier (5).

Among the potentially neurotoxic agents with antineoplastic profiles, oxaliplatin, bortezomib, and epothilone-B are commonly used in daily practice, for which neurotoxicity may not only be dose-limiting, but also influence the patients’ quality of life. Hence, utilizing a representative model to predict the neurotoxicity of drugs would be ideal, which would enable us to administer the right drug to the right person by taking the risk of neurotoxicity into account.

Different models have been employed to study the neurotoxicity of anti-cancer agents, including dorsal root ganglia of rats, cerebral ganglia of snails, and many in vitro models such as neurite outgrowth. Given the high burden of this adverse event, development of appropriate assessment tools is essential for early characterization of the drug-induced neuropathy and thus to improve the quality of life of cancer patients. Although still far from an ideal model, we could successfully evaluate the neurotoxic effects of oxaliplatin, bortezomib, and epothilone-B in PC12 rat pheochromocytoma cells after neuronal differentiation (6). Having evaluated the potential efficacy of amifostine in reducing the risk of neuropathy due to these three drugs, we found it up to 50% protective only in the oxaliplatin-treated cell lines. This model has previously been established to study the potential of amifostine to protect against cisplatin-, paclitaxel-, and vincristine-induced neurotoxicity (7). The neurotoxicity of the three

---

**Table 11.1. Neurotoxicity associated with chemotherapy drugs**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Approved application</th>
<th>Target(s)/Nerve Type</th>
<th>Clinical Presentations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bortezomib</td>
<td>Mantle cell lymphoma and multiple myeloma</td>
<td>Mitochondria, endoplasmic reticulum/ sensory nerves</td>
<td>Distal painful paresthesia</td>
</tr>
<tr>
<td>Platinum agents (Cisplatin)</td>
<td>Advanced bladder cancer, ovarian cancer, lung cancer, and testicular cancer</td>
<td>Dorsal root ganglion/ sensory large fibers</td>
<td>Numbness, diminished of proprioception, loss of deep tendon reflexes, loss of vibratory sense, Lhermitte symptom, ototoxicity, coasting effect</td>
</tr>
<tr>
<td>Platinum agents (Oxaliplatin)</td>
<td>Advanced colorectal cancer</td>
<td>Dorsal root ganglion, sodium ion channels/ sensory large fibers</td>
<td>Acute neuropathy, chronic neuropathy, oropharyngeal alldynia, cold hypersensitivity,</td>
</tr>
<tr>
<td>Vinca alkaloids (vincristine, vinblastine, vindensine)</td>
<td>Acute leukemia, rhabdomyosarcoma, neuroblastoma, Wilm's tumor, Hodgkin's disease, and other lymphomas</td>
<td>Dorsal root ganglion, microtubules, nerve terminals/ sensorimotor small fibers, autonomic and cranial nerves</td>
<td>Dizziness, ocular palsy, facial weakness, vocal cord paralysis, numbness, pain, extensor weakness, constipation, orthostatic hypotension and syncope</td>
</tr>
<tr>
<td>Taxanes (docetaxel, paclitaxel)</td>
<td>Breast cancer, head and neck cancer, gastric cancer, hormone-refractory prostate cancer, and non small cell lung cancer</td>
<td>Dorsal root ganglion, microtubules, nerve terminals or channels/ sensorimotor large and small fibers</td>
<td>Painful paresthesia, myalgia</td>
</tr>
<tr>
<td>Thalidomide</td>
<td>Multiple myeloma</td>
<td>Dorsal root ganglion, nerve blood supply, dysregulation of neurotrophin/ sensorimotor nerves</td>
<td>Painful paresthesia, myalgia</td>
</tr>
</tbody>
</table>

**Note:** Almost all signs and symptoms are usually reversible with dose reduction or cessation of the responsible drug.
drugs was shown by a significant reduction in neurite outgrowth without diminishing the drug cytotoxicity, among which coincubation and 1-hour, not 24-hour postincubation of amifostine could prevent paclitaxel and cisplatin neurotoxicity, respectively. Thus, amifostine had no neuroprotective property against vincristine-induced neurotoxicity being caused by breakdown of neurotubules in the axon. Verstappen et al (7) postulated that vincristine has, presumably, no effect on the neuronal cell itself. Amifostine is thought to scavenge free radicals (8), bind to platinum (9) and alkylating agents (10) and lead to secondary removal of preformed DNA-adducts (10) in cisplatin-induced toxicity and presumably also in cisplatin- and paclitaxel-induced neurotoxicity.

Cyclins B1 and B2 are two known activators of Cyclin-dependent kinase 1 (CDK1) operating during mitosis in human cells, which prevents DNA re-replication (11). Cyclins B2 has been found to be upregulated in many human cancers with a significantly higher relative expression of circulating Cyclins B2 mRNA in cancer patients compared to normal controls and benign diseases group (12). Moreover, circulating Cyclins B2 mRNA level was significantly associated with cancer stage and metastasis status and thus can have potential clinical applications in screening and monitoring of metastasis and therapeutic treatments (12;13). This overexpression may be independent of the gene amplification and lead to the chromosomal instability of cancerous cells (14). Cyclins B1 and B2 as well as p53 have been also involved in apoptosis and differentiation of symptomatic neurons, oligodendrocytes and PC12 cells (15-17). Likewise, Baculoviral Inhibitor of apoptosis Repeat Containing 5 (Birc5) is a multitasking protein that has dual roles in promoting cell proliferation and preventing apoptosis, which is overexpressed during fetal development and in most tumors.

Neurite outgrowth plays a key role in neuronal development and regeneration, and is the hallmark assay for the effects of neurotrophic factors such as nerve growth factor (NGF). NGF has been suggested to trigger neuronal differentiation, followed by neurite outgrowth (18). However, measuring neurite outgrowth is a slow and resource-intensive process. Oe et al (19) suggested Cyclins B2 and Birc5 as surrogate biomarkers for measuring NGF-related neurite outgrowth. Nevertheless, association of neurotoxicity of anticancer agents with dysregulation of these critical factors has never been evaluated. Our results showed that downregulation of Cyclins B2 mRNA may predict oxaliplatin neuronal neurotoxicity (6), and it can be used as a predictor marker. Birc5 mRNA expression was not significantly different than the controls, thus we did not have enough evidence for or against its predictive value (6). Our outcomes prompt future studies to test neurotoxicity of novel agents before starting the treatment. Accordingly, before coming to a decisive conclusion whether or not these findings can be utilized into practice, our results need to be replicated in an independent study.

Many treatments have been tested in clinic to prevent chemotherapy-induced peripheral neuropathy, none, however, have demonstrated consistent efficacy with respect to side effects in clinical trials to be recommended for routine administration (20;21). Calcium and magnesium infusion, selective serotonin and serotonin-norepinephrine reuptake inhibitors, vitamin E, etc. are regarded effective in terms of targeting proposed mechanisms of chemotherapy-induced peripheral neuropathy. They are, despite lacking robust evidence, used in clinical practice as off-label options in order to decrease the risk and/or severity of platinum-induced peripheral neuropathy. Some hypotheses are proposed, which may explain the inconsistency of drug efficacy. The environment is one of the major factors that impact the cellular pumps, channels, transporters, and isoenzymes to some degree, and they are all involved in controlling cellular pH and vice versa (22).

Therefore, the pH variability in the nervous system may influence the drug effects in the nervous system. Both the 2014 Cochrane review and the guideline of American Society of Clinical Oncology did not recommend any of the agents available for the prevention of chemotherapy-induced peripheral neuropathy (Table 11.2) (20;21).

Hence, a critical appraisal of the fundamental data is needed to reassess and analyze current knowledge of the mechanism of drug-induced neurotoxicity. In addition, further basic research is also
necessary to fully understand how we can improve the potency of cytotoxic agents, while protecting the peripheral nerves from the toxicity of these agents. Furthermore, careful design of the clinical trial on the basis of thorough review of the literature as well as patient selection and appropriate methodology may help to avoid unneeded waste of time, energy, and money (23).

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Strength of recommendation</th>
<th>Strength of evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcysteine</td>
<td>Inconclusive</td>
<td>Low</td>
</tr>
<tr>
<td>Acetyl-L-carnitine</td>
<td>Strongly against</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Amifostine</td>
<td>Moderately against</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>Moderately against</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Calcium/magnesium infusion</td>
<td>Moderately against</td>
<td>High</td>
</tr>
<tr>
<td>Carbamazepine/oxycarbazepine</td>
<td>Inconclusive</td>
<td>Low</td>
</tr>
<tr>
<td>Diethyldithiocarbamate</td>
<td>Strongly against</td>
<td>Low</td>
</tr>
<tr>
<td>Glutamate/glutamine</td>
<td>Inconclusive</td>
<td>Low</td>
</tr>
<tr>
<td>Glutathione for paclitaxel/carboplatin</td>
<td>Moderately against</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Glutathione for cisplatin or oxaliplatin</td>
<td>Inconclusive</td>
<td>Low</td>
</tr>
<tr>
<td>Goshajinkigan (Kampo medicine)</td>
<td>Inconclusive</td>
<td>Low</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>Strongly against</td>
<td>Low</td>
</tr>
<tr>
<td>Omega-3</td>
<td>Inconclusive</td>
<td>Low</td>
</tr>
<tr>
<td>Org 2766</td>
<td>Moderately against</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>Moderately against</td>
<td>Low</td>
</tr>
<tr>
<td>rhuLIF</td>
<td>Moderately against</td>
<td>Low</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>Insufficient</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>Moderately against</td>
<td>Intermediate</td>
</tr>
</tbody>
</table>

Note: This table summarizes the data about the strength of evidence for or against the clinical application of the referenced agent in preventing chemotherapy-induced peripheral neuropathy in order to help clinical interpretation (adapted for the 2014 American Society of Clinical Oncology Clinical Practice Guideline). Overall none of the agents showed robust and consistent evidence to be recommended in the practice (chapter 2).

Part 2: Role of signaling in the efficacy of cancer treatment

Despite considerable success in cancer treatment, therapy resistance usually compromises long-term survival. Accumulating evidence shows that the DNA-targeted therapies, as a large category of antineoplastic agents, may significantly modulate key signaling pathways. Complex signaling networks continuously affect the extracellular and intracellular pathways in order to regulate cellular behavior, such as growth, proliferation, migration, or death. This network has been described earlier as signal-cue-response model, which explains how stimulants govern the signaling network and thus cell behavior (24). Alterations to this network due to any reason may change the gene expression and are commonly seen in cancer cells. The PI3K/Akt signal transduction pathway, a survival pathway, controls most hallmarks of cancer. This pathway is widely implicated in tumor growth and causes resistance to
treatment. Activation of the Akt cascade promotes malignant phenotype and is also widely implicated in drug resistance. Therefore, modulation of Akt activity, as one critical pathway, is regarded as an attractive strategy to enhance the efficacy of cancer therapy and irradiation. We reviewed (chapter 5) the current evidence on the efficacy of adding a PI3K/Akt inhibitor to the traditionally used DNA-targeted agent for specific cancer types in vitro, in vivo, and in the clinic. However, overall despite some suggestive evidence in favor of the improving the cytotoxicity of cancer treatment, the heterogeneity of the data deter against advising any combination which would significantly benefit the conventional anticancer regimens.

Because of the treatment resistance that patients develop against standard platinum drugs, we evaluated the efficacy of satraplatin. Moreover, this compound can be given orally. Our results (Chapter 8) demonstrate that the combination of JM118 (active metabolite of satraplatin) and the epidermal growth factor receptor (EGFR) inhibitor erlotinib, showed a schedule dependent synergism in a panel of non-small cell lung cancer (NSCLC), ovarian, and colon cancer cell lines with different molecular properties. Simultaneous exposure was least effective in most cell lines, while JM118 followed by the combination of erlotinib and JM118 was the most synergistic schedule, even in cells with a Ras mutation, by which the expression of EGFR ligands is stimulated and leads to downstream signaling through phosphorylated Erk1/2 and Akt (25-27). Moreover, the amount of platinum-adduct formation, cell kill, and Akt/Erk phosphorylation had positive correlations with the synergistic effect. Besides, the efficacy of erlotinib was cell line dependent, possibly due to their different properties. In wild type cells, especially with activating mutations, erlotinib has an inhibitory effect on phosphorylation of EGFR and downstream proteins [54, 55], while with EGFR/Ras mutated genes, these effects are not expected. It is postulated that activating EGFR mutations result in the dominance of the EGFR, rather than other receptors, pathway in survival and thus more sensitivity to EGFR inhibitors (28). Conversely, cells with intact EGFR may depend mainly on several other receptors that control cell survival through activation of Akt and ERK1/2 pathways (29;30); hence, the cells become more resistant to EGFR inhibitory drugs. Based on these data and our findings, the effect of erlotinib is sufficient to inhibit DNA repair pathways as can be concluded from the increased platinum-DNA adduct formation and cell kill. This was because the combination could, in parallel, inhibit cell cycle proteins and survival pathways, which was also reported for oxaliplatin (31). The synergistic effect of the combination regimen resulted in an increased accumulation of platinum adducts in the DNA, and thereby enhanced the disruption of the cell signaling pathways, which led to growth inhibition. Consequently, preincubation with JM118 followed by the JM118/erlotinib seems the most cytotoxic schedule for the combination of two oral agents, which is worth further investigations.

Identification of genes that harbor highly correlated SNPs is critical, because they usually result in large changes in gene expression in tumor versus normal tissue. Genome-wide association studies (GWAS) have reformed human genetics and can potentially impact human health (32). By the means of GWAS, a myriad of loci have been identified that foster risk stratification to diseases (33;34), and led to better understanding of the genetic architecture of complex traits in the hopes of moving closer to a full molecular characterization of the genetic contributions to cancers, as one area of interest for its usage. However, to minimize the false positive rates, ideally the results are urged to accompany independent replication and verification (35). Furthermore, additional analysis is always needed to understand the functional significance of the identified SNPs in the post-GWAS era (36).

Different tag SNPs have been found to directly or indirectly associate with higher risk and/or severity of varying cancers (37-41) and thus compromise the final outcome. Comorbidities, such as cachexia may also further negatively influence the clinical outcome and quality of life of cancer patients (42-44) and thus correlate with the risk of associated SNPs. Pancreatic ductal adenocarcinoma (PDAC) patients have the highest risk of developing cachexia, which is a direct cause of reduced quality of life and shorter survival. Increased levels of cytokines, such as IL-6, were associated with weight loss and poor prognosis in PDAC patients (45-47). A comprehensive pharmacogenetic study demonstrated an association of cachexia with the SELP-rs6136 polymorphism in roughly 1800 cancer patients (48). Since
apoptosis is reported to take place in wasting muscle in cancer cachexia, several other studies evaluated the key role of Akt1 in developing cachexia (49-51). Akt1 plays a pivotal role in protein synthesis and protein degradation, which contribute to excessive muscular loss in many systemic diseases (52). Thus, we studied the association of cachexia with the three of the most suggestive factors according to the literature in a homogeneous setting of patients with chemo naïve advanced pancreatic cancer in two independent populations.

The results presented in chapter 9 suggest an association between cachexia and the SELP-rs6136-AA and AKT1-rs1130233-AA/GA genotypes in two independent cohorts. We postulate that AKT1-rs1130233 polymorphism may predict the risk of cachexia and death in patients with locally advanced or metastatic PDAC. In a systematic study on genetic determinants of cachexia (53), SELP-rs6136-AA was the only significantly correlated with the risk of cachexia among 129 polymorphisms in 80 genes. In contrast, despite the increased susceptibility of the IL6-rs1800796-GG genotype patients to developing cachexia with decreased survival time in patients with stage II and III PDAC in a Chinese population (54), we could not find a statistically significant correlation. These discrepancies could suggest that pharmacogenetic associations are not always reproducible in studies with populations with different ethnic backgrounds and/or different clinical stages. Accordingly, larger studies with homogeneous settings of patients are essential to investigate the role of emerging biomarkers before planning of prospective trials.

Conclusion

Different tools have been developed to evaluate chemotherapy-induced peripheral neurotoxicity, to measure its severity and to investigate the neurotoxic effects of drugs. Biomarkers may help to predict the risk and severity of drug-induced neurotoxicity. Moreover, the validity and reliability of these markers need to be replicated and also carefully studied in pilot clinical setting. Platinum derivatives harm peripheral nerves and dorsal root ganglia, possibly because of progressive DNA-adduct accumulation and inhibition of DNA repair pathways. Therefore, careful individual selection of appropriate neuroprotective agents may help to reduce neurotoxicity caused by chemotherapeutic agents and thus improve the profile of the treatment in cancer patients. In addition, employing the best available evidence in the clinic along with extensive collaboration between scientists and health professionals may help to elucidate a breakthrough in cancer treatment that would also improve the patients’ quality of life. Besides, preclinical models, surrogate biomarkers, and potentially related gene studies can be valuable to investigate the pathogenesis of drug-related (e.g. neurotoxicity) and disease-related (e.g. cachexia) side effects, and/or possibly predict the associated risks. Furthermore, searching for the most effective schedule for combining newer targeted drugs with conventional regimens is critical in cancer management. In other words, simultaneous administration of two potentially effective drugs will not necessarily show synergism, while preincubation with one of the two can improve the cytotoxicity. Current evidence provides many leads for further investigations to optimize the cytotoxicity of conventional antineoplastic agents, but still many questions have remained unanswered with regard to pathogenesis of chemotherapy-induced peripheral neurotoxicity and role of signaling in improving the efficacy of cancer treatment.

References


CHAPTER 12

Summary,
Curriculum Vitae,
Acknowledgements
Summary

Neuropathy and resistance to the treatment are two main burdensome consequences of chemotherapy. Neurotoxicity is the most common dose-limiting side effect of platinum compounds, which often leads to treatment withdrawal, affects the patients’ quality of life, and sometimes is irreversible. **Chapter 1** shortly reviews the history of the conventional and emerging platinum-based drugs, and the characteristics of the common platinum agents in cancer treatment. More in depth, **chapter 2** discusses available evidence on the pathogenesis and pathophysiology of platinum-induced peripheral neurotoxicity and discusses the characteristics and tools for its timely diagnosis. This chapter also summarizess available evidence on neuroprotective and therapeutic strategies, which indicates the heterogeneity and inconsistency of the data. Accordingly, the evidence does not clearly support the advantage of any of the tested agents to be utilized for affected patients with respect to their safety and efficacy.

Identification and quantification of adverse outcomes associated with exposures to chemotherapy agents, however, has remained widely undetermined, and is still under investigation, and numerous challenges still exist in translating biomarker research into the clinical practice. Different models have been introduced to study chemotherapy-induced neurotoxicity. The results in **Chapter 3** supports the importance of the neurite outgrowth in PC12 rat pheochromocytoma cells for evaluation of the neurotoxicity. With this method, we demonstrated the neurotoxicity of oxaliplatin, bortezomib, and epothilone-B, and investigated the neuroprotective effect of amifostine. Besides, we found that upregulation of cyclin-B2 mRNA was associated with neuronal differentiation and thus oxaliplatin-induced neurotoxicity. Hence, cyclin-B2 can be used as a predictive marker.

Calcium and magnesium infusion is one of the commonly used off-label strategies by oncologists in the hope of preventing or at least, to some extent reducing the severity of oxaliplatin-induced peripheral neurotoxicity. However, consistent evidence is lacking for this method, and recent data did not support the efficacy of calcium/magnesium infusion in preventing this side effect. **Chapter 4** shortly discusses this method and some tools to measure its efficacy, as well as the importance of employing suitable methodology in analysis the results.

Activation of the Akt-survival pathway is a mechanism of resistance to DNA-targeted drugs. **Chapter 5** reviews the effect of common DNA-targeted anticancer drugs on Akt pathway, to see whether antagonizing the Akt survival pathway acts synergistically with the conventional treatments. Available data suggest that the mechanism of drug resistance is complex and comprises difference pathways. There is evidence in favor to improve the cytotoxicity of cancer treatment. However, the available preclinical data is so heterogeneous (varying with drugs) and clinical evidence is limited.

There is evidence of a constitutive activation of Akt in pancreatic cancer. **Chapter 6** describes the therapeutic potential of the novel Akt inhibitor perifosine in combination with gemcitabine in pancreatic ductal adenocarcinoma (PDAC) cells. Perifosine could effectively interfere with cell proliferation, induce apoptosis, reduce migration/invasion, and synergistically interact with gemcitabine in cells with phospho-Akt overexpression.

Increasing the formation and retention of platinum-DNA adducts by decreasing the DNA-repair enzymes may boost the antineoplastic effects of the treatment and overall survival. **Chapter 7** explores the importance of protein/mRNA expression-analysis, as well as the role of polymorphism in the DNA repair enzymes in non-small cell lung cancer and PDAC.

Satraplatin is the only oral analog among platinum agents, which in case of success, may significantly decrease the burden of parenteral cancer therapy. **Chapter 8** discusses our results with
regard to the potential synergism between erlotinib, an epidermal growth factor receptor inhibitor, and JM118, the active metabolite of satraplatin. The synergistic effect of the combination regimen resulted in an increased accumulation of platinum adducts in the DNA, and thereby enhanced the disruption of the cell signaling pathways, which led to growth inhibition. Consequently, we found that preincubation with JM118 followed by the JM118/erlotinib could improve the profile of the two oral agents.

Single nucleotide polymorphisms may be indicative of a shorter survival. Chapter 9 explores the role of novel predictive biomarkers for cachexia, as a direct cause of reduced quality of life and shorter survival, in PDAC. In locally advanced and metastatic PDAC, AKT1-rs1130233 and SELP-rs6136 polymorphisms were associated with the risk of developing cachexia. Moreover, AKT1-rs1130233-AA/GA genotypes were significant predictors for shorter survival, with an increased risk of death in two independent cohorts. We also found a correlation with reduced phosphorylation of Akt1 in muscle biopsies from patients harboring these particular Single nucleotide polymorphisms. Thus, it may play a prognostic role, which needs to be replicated in another independent study before utilizing it into the clinic.

Single nucleotide polymorphisms may predict brain metastases in non-small-cell lung cancer. Chapter 10 discusses some controversies between our findings and some recent data in the role of single nucleotide polymorphisms of Akt in predicting brain metastases in non-small-cell lung cancer.

Chapter 11 discusses the results presented in the present thesis compared with others in the literature. Current evidence provides many leads for further investigations to optimize the cytotoxicity of conventional antineoplastic agents, but still many questions remain unanswered with regard to the pathogenesis of chemotherapy-induced peripheral neurotoxicity and role of signaling in improving the efficacy of cancer treatment, which warrants more studies.
Chapter 12 (Avan’s dissertation)

Samenvatting

Neurotoxiciteit en resistentie tegen een behandeling zijn twee mogelijke complicaties bij de behandeling van patiënten met kanker. Neurotoxiciteit is een van de meest voorkomende bijwerkingen van platinaverbindingen. Dit leidt vaak tot het stoppen van de behandeling en een afname in de kwaliteit van leven. De toxiciteit is soms onomkeerbaar. Hoofdstuk 1 geeft een kort overzicht van platinaverbindingen, waaronder zowel het klassieke cisplatin als nieuwe platinaverbindingen, en hun rol in de behandeling van kanker. Hoofdstuk 2 geeft een overzicht van de beschikbare informatie over hoe platinaverbindingen neurotoxiciteit veroorzaken en wat de kenmerken van deze toxiciteit zijn. Dit is van belang bij het stellen van de diagnose. In dit hoofdstuk worden naast de behandelmogelijkheden ook de verschillende strategieën om neurotoxiciteit tegen te gaan samengevat. Er is een grote heterogeniteit in neurotoxiciteit, terwijl de beschrijving van deze toxiciteit vaak inconsistent is. Dat maakt het moeilijk om beschikbare middelen die relevant bijwerkingen en effectiviteit goed te kunnen beoordelen.

Het identificeren en kwantificeren van neurotoxiciteit van chemotherapie is nog steeds niet goed gevalideerd, zodat er nog veel uitdagingen zijn om biomarkers te vertalen naar de kliniek. Er zijn verschillende modellen beschreven. In Hoofdstuk 3 worden de voordelen van de neuritiene uitgroei methode beschreven. Met dit modellsysteem is de neurotoxiciteit van oxaliplatin, bortezomib en epothilone B onderzocht. Ook het omkeren/beschermen tegen neurotoxiciteit door amifostine is nader bekeken. Als een alternatieve marker voor neuronale differentiatie is de verhoging van cyclin B2 mRNA expressie bestudeerd.

Calcium en magnesium infusen worden vaak off-label (oneigenlijk) gebruikt om perifere neurotoxiciteit te voorkomen of te verminderen. Het probleem hierbij is dat de resultaten tot nu toe niet overtuigend zijn. Deze methode wordt in Hoofdstuk 4 beschreven, waarbij ook het belang van goede assays benadrukt wordt.

Activering van de Akt overlevingsroute is een resistentie mechanisme tegen DNA gerichte geneesmiddelen. In Hoofdstuk 5 worden de effecten van een aantal van de geneesmiddelen (platinum anaaloga, gemcitabine, taxanen, antifolaten) op deze route beschreven. De hypothese is dat de effectiviteit van DNA-gerichte middelen versterkt kan worden door de AKT route te remmen.

Hoofdstuk 6 beschrijft de rol van AKT activatie in resistentie van alvleesklier kanker tegen standaard behandeling met gemcitabine. Verder is de potentie van perifosine, een AKT antagonis, in combinatie met gemcitabine onderzocht. Perifosine remde de cellulaire proliferatie, induceerde apoptose en remde de migratie/invasie en van tumor cellen. In cellen met een hoge expressie van fosfor-AKT was perifosine synergistisch met gemcitabine.

De vorming van platina-DNA adducten is cruciaal voor de antitumor activiteit van het antikanker middel cisplatin. Deze adducten kunnen hersteld worden door DNA repair enzymen, waardoor de effectiviteit van cisplatin verminderd wordt. Een lage activiteit van deze enzymen zal dan ook de aanwezigheid van deze adducten in de tumorcellen verlengen en daarbij de effectiviteit van cisplatin verhogen. In Hoofdstuk 7 beschrijven we het belang van de eiwit en RNA expressie van DNA-repair enzymen. Ook werden polymorfismes in DNA repair enzymen in long kanker en alvleesklier kanker onderzocht.

Satraplatin is een nieuw platina analoog wat oraal gegeven kan worden. Hoofdstuk 8 beschrijft synergie van JM118, de actieve metaboliet van satraplatin, met erlotinib, een remmer van de epidermale groei factor receptor. In de combinatie werd een hogere concentratie van DNA platina adducten gevonden en een verstoord reguleren van signaaltransductieroutes. Dit leidde tot een
verhoogde groeiremming. Verder vonden we dat een voorbehandeling met JM118 gevolgd door de combinatie JM118/erlotinib het beste groeiremmende effect had.

Single nucleotide polymorphisms (SNP’s) zijn veranderingen in een bepaalde base in het DNA. SNP’s kunnen het effect van een behandeling voorspellen. In Hoofdstuk 9 beschrijven we de rol van bepaalde SNP’s in de aanwezigheid van cachexie, een versterving van normaal weefsel, waardoor een patiënt snel gewicht verliest, diens kwaliteit van leven achteruit gaat en sneller overlijdt. Cachexie is een van de kenmerken van alvleesklier kanker; de SNP’s in het AKT1 gen (rs1130233) en het SELP gen (rs6136) bleken gerelateerd te zijn aan het ontwikkelen van cachexie. Het AKT1 gen (rs1130233) AA-CA genotype had in twee aparte groepen patiënten een significante relatie met een verkorte overleving en verhoogd risico op overlijden. We vonden ook een correlatie tussen deze SNP’s en een verlaagde fosforylering van AKT1 in spierbiopten van patiënten. SNP’s hebben wellicht een voorspellende waarde, maar dit dient eerst in een grotere studie aangetoond te worden.

SNP’s kunnen ook voorspellen of patiënten met longkanker hersenmetastases ontwikkelen. In Hoofdstuk 10 wordt de rol van bepaalde SNP’s in het AKT gen in de vorming van hersenmetastases van longkanker besproken.

In Hoofdstuk 11 worden de resultaten uit dit proefschrift vergeleken met die in de literatuur. Er zijn vele mogelijkheden om de effectiviteit van conventionele cytotoxische middelen te verbeteren. Er is echter nog steeds veel onduidelijkheid over het ontstaan van perifere neuropathie en bij hoeveel patiënten dit tot complicaties leidt. Ook de vraag hoe het beïnvloeden van signaaltransductieroutes de effectiviteit van therapie kan verbeteren, moet verder onderzocht worden.
فصل اول این پایان نامه بطور خلاصه تاریخچه و ویژگی‌های دارویی قدرت و جدیدتر داروهای با ترکیب پلاتین و موارد مکنیکی

فصل دوم به طور جامع شواهد علمی موجود را در زمینه علیه و مکانیسم اسبی عصب‌های محیطی به دلیل پلاتین را مورد بررسی قرار داده و ویژگی‌های اختصاصی یال‌زنبور و روش‌های مورد استفاده برای تشخیص شناسایی زیان آن تایژ می‌کند. این فصل محتویاتی شامل اطلاعات مختلف است. در نتیجه، در حال حاضر شواهد علمی مستند و کافی برای تایژ هیچ گاهی از این اقدامات.

پیشگیری کننده میژود نیست تا بتوان استفاده از آن را با محدودیت در مورد این اختلال توصیه کرد.

هرچند استفاده بالینی از نشانه‌های پاراکلینیک و پیامدهای نوین مرتبط با اختلال با بیماری مورد بررسی بسر به مشکل است.

ترزیک ترکیب کلسیم منیزیمی یکی از روش‌های مورد توجه متخصصین خون و سرطان شناسی بوده که به جهت پیشگیری و یا کاهش شدت اثرات داروهای آکزیتلاتین بر روی عصب مورد استفاده قرار می‌گرفته است. هرچند نتایج تحقیق‌ها هم راستا با هم نیستند و مطالعات اخیر نیز اثر ترکیبی آن را زیر سوال برده است. فصل پنجم کتاب بطور خلاصه این روش درمانی ارزیابی اثر‌بخشی آن و اهمیت استفاده از روش مناسب در آنالیز باقیه ها و مورد بحث قرار می‌دهد.

افزایش فعالیت مسیر اکت‌ها در بدن می‌تواند باعث شود متابولیسم داخلی یکی از داروهایی که به عنوان پیشگیری و یا کاهش شدت اثرات داروهای موجود یا اثرات داروهای مورد استفاده قرار می‌گرفته است. هرچند نتایج تحقیق‌ها هم راستا با هم نیستند و مطالعات مختلف مختلف به دست آمده و در نتیجه نمی‌توان به یک نتیجه گیری و توصیه واحد برای استفاده از این مسارکنده‌ها در کنار داروهای قدرتمند.
پایتخت به شواهد افزایش فعالیت مسیر اکت در سرطان پانکراس، در فصل پنجم، بانکس درمانی پریفورسین به عنوان مهارکننده مسیر اکت در کنار دارویی جمسایتوئین "مودر برسی و ازمایش قرار گرفته است. نتایج حاصل از این تحقیق نشان داد پریفورسین می‌تواند در پروابد سلولی که کیفیت را کاهش دهد و می‌تواند طیف باعث انرژیات اثر ضد سرطانی جمسایتوئین در سلول‌های با افزایش هسته‌ای گردد. علاوه بر مکانیسم فوق، سرکوب مسری ترمیم رنگی با افزایش تشکیل کمپلکس پلنکتی در این ای جز می‌تواند اثر بخشی درمانی‌های ضد سرطان و طول عمر بیماران را افزایش دهد که در فصل هفتم مورد مطالعه قرار گرفت. و اهمیت بیان پرتوپتین، برخی اینها ها از انتخابات رنگی در انسیم‌های ترمیم کننده تر در سرطان ریز و پانکراس برسی و گردد.

سانتاپلاتوئین می‌تواند در مقایسه با داروهای تریپائیدی متداول جایگزین مناسبی در درمان بسیاری از سرطان‌های باشد. در تحقیقات ارائه شده در فصل هشتم نشان داده شد که ارزش‌های مهارکننده غشایی مانند در کنار سانتاپلاتوئین اثر ضد سرطانی بهتری دارد که مکانیسم آن نیز مورد مطالعه قرار گرفت.

وجود جهش‌های نطفه ای خاص در برخی از زن‌ها ممکن است با طول عمر کنترل بیماران سرطانی در اربت باشد. در فصل نهم، نقش برخی از فاکتورهای بیش بینی کننده کاهش‌کننده (لاگری‌های با تحمل غضروفی) که با طول عمر کنترل بیماران سرطانی آن‌ها است. این بناست در دور مطالعه مستقل مورد مطالعه قرار گرفت. به طور مشابه، وجود جهش‌های نطفه ای خاص در سرطان‌های ممکن است شناساند است. انسان به مغز را پیش به‌ند که به‌طور خاص با فصل دهم مورد بحث قرار گرفته اند.

در فصل یازدهم، مجموعه‌ای از نتایج این تحقیقات مورد بحث قرار گرفته است. با وجود بی‌پرتوپتینی‌های مختلف در درمان انسان و نتایج حاصل از مطالعه‌های بحث قرار گرفته‌ها و همچنین نامه‌هایی که از دانش‌های‌ها ما در زمینه سرطان بیشتر است و سؤالات بسیاری در مورد عوارض عصبی و مقاومت دارویی بیماران ها و درمان سرطان‌بی‌پرتوپتین است که به‌طور معمول نشان‌دهنده است که نیازمند تحقیقات بیشتر و دقیق تر در این زمینه است.

perifosine
gemcitabine
apoptosis
phospho-Akt
platinum-DNA adducts
satraplatin
erlotinib
epidermal growth factor receptor inhibitor
synergistic effect
cachexia
**CURRICULUM VITAE**

**Abolfazl Avan**

**Profile**

Abolfazl Avan was born on the eighteenth of February 1982 in Mashhad, Iran. He studied at Azarmanesh Primary School from 1988 to 1993 (5 years), Din-o-Danesh Secondary School from 1993 to 1996 (3 years), Allameh High School from 1996 to 1999 (3 years), and Sama Colleague from 1999 to 2000 (1 year). He has been the top first or second student during his 12-year preuniversity education period with grade point average of higher than 19 out of 20. He was ranked among the top 0.1% candidates in the National University Entrance Exam and granted the 7-year medical scholarship. He studied medicine at Tabriz University of Medical Sciences from 2000 to 2006 and did his one and a half years clinical internship as well as his medical doctorate thesis on malignant mesothelioma at Mashhad University of Medical Sciences from 2007 to 2009. Abolfazl earned his medical doctorate from the sixth top national university in November 2009. Later, he worked at the Emergency Department for about two years thereafter. Besides, he has been actively cooperating with neurologists and gastroenterologists, particularly in treating Wilson’s disease patients since 2009. He also had different presentations at major national universities and an international congress. Abolfazl has made an international network with worldwide seniors, which led to receiving the Free Copper Prize in 2012, and a grant from the Movement Disorder Society in 2013. After about 4 years of clinical practice and research, he moved to the Netherlands to do a PhD on pathogenesis of neurotoxicity and role of signaling in the efficacy of anticancer agents at VU University Medical Center in Amsterdam under the supervision of Prof. Dr. Godefridus J. Peters, PhD and Dr. Elisa Giovannetti, MD, PhD. In July 2015, he was invited to collaborate with Dr. Mahmoud Reza Azarpazhooh and a Canadian team on a systematic review on neuropathology of dementia under the supervision of Prof. Vladimir Hachinski, MD at the Western University in London, Ontario, Canada. Furthermore, he joined a clinical project on eye movement disorders in Parkinson’s disease and essential tremor under the supervision of Dr. Anne-Fleur van Rootselaar, MD, PhD at the Department of Neurology in the Academic Medical Center of the University of Amsterdam. He is also a junior member of the American Academy of Neurology and the International Society of Parkinson’s disease and Movement Disorders.

**Experiences**

- **Department of Neurology and Neurophysiology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands.** July 2015-Now  
  Eye movements disorders in Parkinson’s disease and essential tremor  
  (Supervisor: Dr. A.F. van Rootselaar, MD, PhD)
- **Cancer Center Amsterdam, Department of Medical Oncology, VU University Medical Center, Amsterdam, the Netherlands.** 2013-Now
- **Collaboration with the Departments of Neurology, Gastroenterology, and Pediatric Gastroenterology, Ghaem Hospital, Mashhad University of Medical Sciences, Mashhad, Iran.** (Clinician, treating Wilson’s disease patients) 2009-2013
• Emergency Department, Dr. Chamran Hospital, Ferdows, Birjand University of Medical Sciences. (Emergency Room Clinician) 2010-2011

Medical Education

- General medicine and clinical traineeships 2001-2006
  Tabriz University of Medical Sciences (TUMS), Tabriz, Iran
  (#6 National Medical University)
- Clinical Internships (GPA: A – 17.16 out of 20) 2007-2009
  - a) Ghaem Hospital, b) Imam Reza Hospital, c) Dr. Sheik Hospital, d) Ebn-e Sina Hospital, Mashhad University of Medical Sciences (MUMS), Mashhad, Iran.
  (#3 National Medical University)
  - a) Imam Khomeini Hospital, b) Sina Hospital, c) Imam Reza Hospital, d) Telaghami Hospital, e) Azzahra Hospital, and f) Shohada Hospital Tabriz University of Medical Sciences (TUMS), Tabriz, Iran. (#6)

Academic Honors

- Awarded the MDS $2,000.00 USD Grant by “The Movement Disorder Society's Award Committee,” 17th International Congress of Parkinson's Disease and Movement Disorders, June 16-20, 2013 in Sydney, Australia. 2013
- Awarded Hoogenraad Prize (Free Copper Prize; The Bronze Owl) by Dr. Tjaard U. Hoogenraad, promotor of zinc therapy since 1978, for my work on Wilson’s disease. 2012
- Invited for the management of patients with Wilson’s disease. Mashhad University of Medical Sciences, Mashhad, Iran. 2011
- Awarded the the Governor General’s honour as an active and compassionate doctor in Dr. Chamran hospital, Ferdows, South Khorasan, Iran. 2010
- Awarded €1,000.00 grant from the Stichting De Kist van Beresteyn (founded in 1606 in the Netherlands) for my works on Wilson's disease; 23 April 2009
- Invited for the management of patients with Wilson's disease. Tabriz University of Medical Sciences, Tabriz, Iran. As clinician and consultant. 2009
- Granted the 7-year medical education National Scholarship from the Ministry of Health and Medical Education, Iran; (ranked 617 among 0.6 million candidates) 2001

Peer-Reviewed Publications

### Peer-Reviewed Publications (cont’d)

- **Ceresa C,* Avan A,* Giovannetti E, Geldof AA, Avan A, Cavaletti G, Peters GJ.** Characterization of and Protection from Neurotoxicity Induced by Oxaliplatin, Bortezomib and Epothilone-B. *Anticancer Res* 2014;34:517-523 [IF:1.83]

### Manuscripts

- **Avan A, Digaleh H, Behrouz R, Azarpazhooh MR, et al.** Exploring the Link between Socioeconomic Deprivation and Stroke. 2015
- **Avan A, Narayan R, Giovannetti E, Peters GJ.** Role of Akt signalling in resistance to DNA-targeted therapy. 2015
- **Avan A,* Maftouh M,* Avan A,* van Krieken A, van Grijzen N, Raktoe R, Funel N, Caponi S, Boggi U, Leon LL, Peters GJ, Giovannetti E.** Phospho-Akt overexpression is prognostic and can be used to tailor the synergistic interaction of the novel Akt inhibitor perifosine with gemcitabine in pancreatic cancer. 2015
- **Avan A, Azarpazhooh MR, Hoogenraad TU.** Free copper intoxication instead of copper accumulation in Wilson’s disease: a critical appraisal. 2015
- **Avan A, Kianifar HR, Hoogenraad TU.** No more liver transplantation: successful zinc therapy in acute liver failure due to Wilson’s disease. 2015

### Post-hoc Review


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Conference proceeding/Poster Presentation (cont’d)

- Avan, A., Kianifar, H.R., Hoogenraad, T.U.; Successful initial zinc therapy in Wilson’s disease with acute liver failure and copper intoxication: Wonder drug exists [abstract]. AD/PD 2015:384 (Nice, France)
- Avan, A., Azarpazhooh, M., Kianifar, H., Hoogenraad, T.; Patients with free copper toxicosis in Wilson’s disease should be treated with zinc from the beginning [abstract]. Movement Disorders 2012;27 Suppl 1:232 (Dublin, Ireland)

Major Trainings

- Movement disorders. CME 2015 – 8 AMA PRA Category 1 Credits; Continuum Audio: (1) Parkinsonian Syndromes and Tremor Syndromes; (2) Chorea and Gait Disorders; (3) Myoclonus and Cerebellar and Afferent Ataxias; (4) Tic Disorders and Psychogenic Movement Disorders.
- Alzheimer’s disease & Dementia: CME 2015 – 8 AMA PRA Category 1 Credits; Continuum Audio: (1) The Diagnostic Evaluation of a Patient with Dementia; (2) Biomarkers of Alzheimer Disease; (3) Nonpharmacologic Treatment and Prevention Strategies for Dementia; (4) Alzheimer Disease Pharmacologic Treatment and Treatment Research; (5) New Diagnostic Schemas, Genetics, and Proteinopathy in the Evaluation of Frontotemporal Degeneration; (6) The Clinical Problem of Neuropsychiatric Signs and Symptoms in Dementia; (7) Dementia in the Oldest Old; (8) Understanding and Treating Vascular Cognitive Impairment; (9) Ethics: When Cognitively Normal Patients Ask to Be Tested for Alzheimer Disease; (10) Test Your Knowledge: Dementia.
- Multiple Sclerosis. CME 2015 – 9 AMA PRA Category 1 Credits; Continuum Audio: (1) Diagnosis and Differential Diagnosis of Multiple Sclerosis; (2) Current and New Directions in MRI in Multiple Sclerosis; (3) Pathology of Multiple Sclerosis: Where Do We Stand?; (4) Present and Emerging Therapies for Multiple Sclerosis; (5) General Health Issues in Multiple Sclerosis; (6) Gait Disorders in Multiple Sclerosis; (7) Optic Neuritis and the Evaluation of Visual Impairment in Multiple Sclerosis; (8) Pediatric Demyelinating Diseases; (9) Disclosing a Misdiagnosis of Multiple Sclerosis; (10) Acute Disseminated Encephalomyelitis, Transverse Myelitis, and Neuromyelitis Optica; (11) Unusual Symptoms and Syndromes in Multiple Sclerosis. NeuroLearn: (12) Sick & Tired: Recognizing and Treating Fatigue in Persons With Multiple Sclerosis.
- Mouse Morphology & Genetics Course. OOA Course, Academic Medical Center,
Amsterdam, the Netherlands, March 23 – April 2, 2015 (72 hours; 3 ECTS points).


- Alzheimer’s disease & Dementia. CME 2014– 5.5 AMA PRA Category 1 Credits: (1) Alzheimer’s Disease Diagnosis and Treatment Update; (2) Practical Approaches to Managing Patients With Moderate to Severe Alzheimer Disease; (3) Case Challenges in Early Alzheimer’s Disease; (4) Emerging Imaging and Therapeutics in Alzheimer’s Disease: Early Detection and Intervention.

- Multiple Sclerosis CME 2014 – 2.15 AMA PRA Category 1 Credits: (1) Hot Topics in Multiple Sclerosis Management: An Expert Discussion; (2) Brain Atrophy, MRI, and Evaluating Oral Therapy in Patients With RRMS; (3) Early Treatment Strategies and Improved Outcomes in RRMS; (4) Nurses and RRMS Management: Real World Experience; (6) The Real-World Evidence for Oral Therapies in RRMS.

- Bioinformatics Sequence Analysis. The PhD course program 2013-2014, AMC Graduate school for Medical Sciences, Amsterdam, the Netherlands

- Histopathology of tumours, OOA Course, Amsterdam, the Netherlands, February 10-11, 2014.

- Basic Medical Statistics (SPSS), OOA Course, Amsterdam, the Netherlands, November 25-29, 2013.

- Movement disorders: Awarded 35 AMA PRA Category 1 Credits. The Movement Disorder Society’s 17th International Congress of Parkinson’s Disease and Movement Disorders at Sydney Convention and Exhibition Centre, Sydney, Australia, June 16-20, 2013.

- Movement disorders: Awarded 35 AMA PRA Category 1 Credits. The Movement Disorder Society’s 16th International Congress of Parkinson’s Disease and Movement Disorders at The Convention Centre Dublin, Dublin, Ireland, June 17-21, 2012.

- Gastroenterology and Hepatology. Awarded 14 CME. 12th Iranian Congress of Gastroenterology and Hepatology, Tehran, Iran, on November 28-30, 2012

- Epilepsy: First International Congress of Epilepsy, Razavi Hospital, Mashhad, Iran. Dec 2010 (7 score CME)

- Internship: Clinical Neurology, Internal Medicine, Paediatrics Medicine, Cardiology, Infectious diseases and Clinical General Surgery at Ghaem Hospital, Imam Reza Hospital, and Dr. Sheikh Hospital. Mashhad University of Medical Sciences. Mashhad, Iran. 2007-2009

- Internship: Emergency Medicine, Orthopaedic Surgery, ENT Surgery, Urology and Radiology at Imam Khomeini Hospital and Imam Reza Hospital. Tabriz University of Medical Sciences. Tabriz, Iran. 2007

- Research Methodology Workshop, Tabriz University of Medical Sciences, Tabriz, Iran. 2004
• IELTS Certificate of English, Score 7.0. 2012 (speaking 7.5)
• English Learning Courses, Certificate of Advanced English (CAE), Allameh English Institute, Mashhad, Iran. 2012
• Violin, Classical Music (In Iran and Holland), 2010-now

Lectures and Presentations
• Back to life: miracle? (Wilson’s disease) In: Main Neurology Meeting at Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands. October 2015
• Wilson’s disease Treatment: First do no harm. In: Gastroenterologists’ meeting at Imam Reza Hospital, Mashhad, Iran. August, 2013.
• Reversal of fulminant Wilson’s disease following two weeks zinc therapy. Iranian International Congress of Gastroenterology and Hepatology. Tehran, Iran. 28 November, 2012
• Evidence Based Decision Making; Effective, Safe and Cheaper Approach in Treatment of a Fatal Disease. Department of Neurology, Mashhad University of Medical Sciences (for neurologists, residents and peers), 2010
• Wilson’s Disease; Prognosis and Differential Diagnoses. Mashhad University of Medical Sciences. Department of Infectious Diseases (for professors, residents and peers), 2009
• Liver Transplantation in Wilson’s Disease; When to Decide. Department of Internal Medicine, Mashhad University of Medical Sciences (For gastroenterologists, residents and peers), 2009
• Decision Making; New Aspect in Treatment of Wilson’s Disease. Department of Internal Medicine, Tabriz University of Medical Sciences (for gastroenterologists, residents and peers), 2008
• The Problem of Wrong Decision. Department of Neurology, Tabriz University of Medical Sciences (for neurologists, residents and peers), 2008
• The Problem of Delayed Diagnosis; Appropriate Approach to Wilson’s Disease. Department of Internal Medicine, Mashhad University of Medical Sciences (for gastroenterologists, residents and peers), 2007
• General overview of Wilson’s disease. Department of Neurology, Tabriz University of Medical Sciences (for neurologists, residents and peers), 2006

Medical Doctorate (MD) Thesis
Evaluation of signs, symptoms, treatment and prognosis of patients with malignant mesothelioma whom were admitted to Ghaem and Omid hospitals from 1982 to 2008
Thesis score: 19.11 out of 20, Grade: “A plus” November, 2009
Supervisors: Dr. Reza Bagheri, MD; Prof. Seyyed Ziaallah Haghi, MD.

Pre-graduation Education
Twelve years of preuniversity education period 1988-2000
GPA: A plus; Scores: higher than 19 out of 20; 1st or 2nd top student
ACKNOWLEDGEMENTS

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Kind appreciation is expressed to the anonymous reviewers of the articles published in this thesis for their precise and critical review, which helped me improve the quality and clarity of my works. Likewise, I am grateful to the official reading committee members for their careful review of my dissertation and their invaluable feedbacks; dr. Jan Buter (Oncologist, VU University Medical Center), dr. Tjaard U. Hoogenraad (Neurologist, University Medical Center Utrecht), dr. Judith R. Kroep (Oncologist, Leiden University Medical Center), dr. Tjeerd J. Postma (Neurologist, VU University Medical Center Amsterdam), prof.dr. Egbert F. Smit (Pulmonary oncologist, Netherlands Cancer Institute and VU University Medical Center Amsterdam), and prof.dr. Thomas Würdinger (Biologist, VU University Medical Center Amsterdam). I hope I can extend my collaborations with all in future. Tjaard has been stimulating me in treating Wilson’s disease patients since 2007 when I saw his monograph on the disease in the library of Tabriz University Medical Sciences, where I was studying medicine. He has always advised me to treat patients with the best available evidence-based patient-oriented scientific approach. I owe him a lot and I am happy to have been working under his precious supervision, as the world-known expert in Wilson’s disease.

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I also had great moments with many Iranian friends in Amsterdam, including Mohammad Ali, Saeideh, Jafar, Leila, Mahdi, Samira, Mohammad, Maryam, Esmail, Elham, Hossein, Zahra, Hodjat, Marzieh, Shayan, Narges, Mohammad Amin, Fatemeh, Hamid Reza, Marzieh, Naser, Aylar, Mahdi, Mahdieh, Masoud, Hoda, Amin, Parisa, Yasser, Hossein, Hadi, Mojtaba, and all those that I might have forgotten to mention. They all made me feel at home. I hope we will keep this fantastic friendship forever.
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Abolfazl Avan

October 2015, Amsterdam