# CHAPTER II
# LITERATURE REVIEW

## 1. Paclitaxel (Taxol®)

Paclitaxel (Taxol®), the first taxane in clinical use, is active against a broad range of cancers that are generally considered to be refractory to conventional chemotherapy. In 1963, a crude extract from the bark of the Pacific yew Taxus brevifolia, was found in preclinical studies to have cytotoxic activity against many tumors. Paclitaxel was identified as the active constituent of this extract in 1971 (Wani et al., 1971).

### 1.1 Antitumor activity

Phase I clinical trials of paclitaxel began in 1981 and were completed after some initial problems due to allergic reactions were solved by premedication and by changing the administration from a bolus injection to a 24 hour infusion. Paclitaxel entered Phase II trials in the mid 1980s, and the first report of clinical activity against ovarian cancer was published in 1989 (Mcguire et al., 1989). This was followed by the report of the activity against breast cancer in 1991 (Holmes et al., 1991). Paclitaxel has been approved for the treatment of drug-resistant ovarian cancer by the Food and Drug Administration (FDA) in 1992. Approval for the treatment of breast cancer was followed in 1994. Clinical use of paclitaxel has increased steadily since then, and it is currently used not only for the treatment of ovarian (Tomita et al., 2001) and breast cancers (Poelman et al., 2000) but also for the treatment of lung cancer (Chu et al., 2005), squamous cell carcinoma of the head and neck (Schrijvers and Vermorken, 2005) and various other cancers (Eisenhauer and Vermorken, 1998; Chang et al., 2003).

## 1.2 Mechanism of action

Paclitaxel stimulates microtubule polymerization (Schiff et al., 1979) by binding directly with high affinity to tubulin along the length of the microtubule. The binding site for paclitaxel is at N-terminal of the β-subunit of tubulin (Rao et al., 1994; Nogales et al., 1995). Binding of paclitaxel to the microtubule increases the polymerization, presumably, by inducing a conformational change in the tubulin resulting in, an increase in the affinity between neighbouring tubulin molecules (Nogales, 2001).

Suppression of microtubule dynamics by paclitaxel leads to mitotic block (Yvon et al., 1999) which causes cell cycle arrest in $G_2$/M phase. Therefore, affected cells cannot proliferate and undergo apoptosis through several mechanisms (Schiff and Horwitz, 1980; Liebmann et al., 1994) such as checkpoint of mitotic spindle assembly, malfunction of cyclin-dependent kinases and change in activity of MAPKs (Sorger et al., 1997; Ibrado et al., 1998; Shen et al., 1998; Wang et al., 1998; Ghatan et al., 2000).

## 1.3 Side effects

### Hypersensitivity Reactions

Most affected patients have type 1 hypersensitivity reactions, which are characterized by dyspnea with bronchospasm, urticaria, and hypotension (Weiss et al., 1990). The frequency and severity are independent of the dose or the schedule of paclitaxel administration. Hypersensitivity reactions typically occur early in the treatment course and sometimes within the first hour of infusion. Anaphylaxis and severe hypersensitivity reactions (hypotension, angioedema and generalized urticaria) occur in 2% of the cases and can be fatal.

**Hematologic Toxicity**

Bone marrow suppression is the major dose-related toxicity of paclitaxel. Neutropenia, the most important hematologic toxic effect of paclitaxel (Rowinsky et al., 1993), is dose-and schedule-dependent and is generally rapidly reversible. The incidence does not appear to increase with cumulative exposure.

**Neurotoxicity**

Neuropathy is the dose-dependent toxicity of several commonly-used chemotherapeutic agents, including paclitaxel (Cavaletti et al., 1995; Quasthoff and Hartung, 2002). The incidence and severity of neuropathy are related to the cumulative dose, and probably to the single-dose intensity as well. Sensory neuropathy, e.g numbness and paresthesia and typically is the most common form of paclitaxel-induced neuropathy (Lipton et al., 1989; Postma et al., 1995) occurs first in a glove-and-stocking distribution in the lower extremities, although fingers and toes may be affected simultaneously (Wiernik et al., 1987; Forsyth et al., 1997). Occasionally, symptoms are initially asymmetric (Lipton et al., 1989) but symmetric distal loss of sensation carried by both large fibers (proprioception, vibration) and small fibers (temperature, pinprick) is more common. The distal, symmetric, length-dependent neurologic deficits suggest that paclitaxel causes an axonopathy. Motor and autonomic dysfunction may also occur, especially at high doses and in patients with preexisting neuropathies caused by, for example, diabetes mellitus and alcoholism (Freilich et al., 1996). The signs and symptoms may continue to worsen for several weeks after discontinuation of the drug, and then improve gradually (Cavaletti et al., 1995 ). Although mild sensory symptoms usually improve or resolve within months after cessation of paclitaxel, severe symptoms may persist for longer than 1 year (Rowinsky and Donehower, 1993; Cavaletti et al., 1995).

Electrophysiologic studies show that paclitaxel reduces both sensory and motor nerve action potential amplitudes. Reduction in amplitudes of sensory nerve and compound muscle action potentials correlates with the cumulative dose (van Gerven et al., 1994). The motor involvement is more likely to be subclinical and occurs later in the course of neuropathy (Freilich et al., 1996).

Histopathologic findings of paclitaxel-induced neuropathy have been reported (Sahenk et al., 1994). Sural nerve biopsy reveals a greater loss of large- than small-diameter myelinated nerve fibers. Axonal degeneration with secondary demyelination and remyelination can be seen. However, regenerating axonal sprouts are uncommon. Electron microscopy has demonstrated accumulation of tubular and membranous structures within the axons (Sahenk et al., 1994).

In pathogenesis, stabilization of microtubule dynamics is believed to be the major cause of paclitaxel-induced neuropathy. Since, axonal transport may be disrupted. This hypothesis is supported by the finding of aggregation and accumulation of abnormal microtubule bundles in dorsal root ganglia, axons, and Schwann cells (Roytta and Raine, 1986). However, more studies are needed to clarify the pathogenesis.

An animal model has been used to study the underlying mechanisms of paclitaxel-induced neuropathy and potential treatments. Sensory disturbances similar to what are seen in patients, are found in the animal model, for example, thermal hyperalgesia in the hind paw (Polomano et al., 2001) and tail (Cavaletti et al., 1997; Polomano et al., 2001). However, some studies also report the development of hypoalgesia in the rats receiving paclitaxel (Campana et al., 1998). Furthermore, decreased nerve conduction velocity has been demonstrated (Cavaletti et al., 1997; Cliffer et al., 1998; Authier et al., 2000; Persohn et al., 2005). Histopathological examination shows axonal

degeneration (Cavaletti et al., 1995; Cliffer et al., 1998; Authier et al., 2000; Persohn et al., 2005) with collapse and fragmentation of myelin sheath (Persohn et al., 2005) including an increase in microtubule density in the axon (Cavaletti et al., 1995; Authier et al., 2000). In addition, Schwann cells with nucleolus-like formation could be found (Cavaletti et al., 1997). Recently, morphometric evaluation of sciatic nerve demonstrates that mean myelinated fiber diameter and number of large myelinated fibers ($ø \geq 10$ μm) are decreased while the number of small myelinated fibers is increased (Persohn et al., 2005). This study also shows that g-ratio (value of the axonal diameter divided by the diameter of the axon with myelin) decreases significantly.

Although neuropathy caused by paclitaxel has been characterized by preclinical and clinical studies as delineated above, the underlying molecular mechanisms are still unclear. Nevertheless, some evidence suggests the possible role of MAPKs in paclitaxel-induced neuropathy. This will be explained in the following section.

## 2. Mitogen-activated protein kinases (MAPKs)

### 2.1 Introduction

Mitogen activated protein kinases (MAPKs) are a family of serine/threonine kinases which have been implicated in cell proliferation, differentiation, and death (Pearson et al., 2001). They are dual specificity enzymes that can phosphorylate hydroxyl side chains of serine/threonine and tyrosine residues in their substrates (Ashworth et al., 1992; Crews et al., 1992; Wu et al., 1993). MAPKs are regulated by phosphorylation cascades (Garrington TP and Johnson, 1999) (figure 1). MAPKs are activated by upstream regulators, MAPK kinases (MAPKKs), in turn, the MAPKKs are activated by MAPKK kinases (MAPKKKs) that transduce signals from

stimulus-activated receptors on the cell surface or through interactions with GTP binding proteins and/or other kinases.

MAPKs at the end of these signaling cascades phosphorylate their target proteins, such as transcription factors, cytoskeletal proteins and other protein kinases (Johnson and Lapadat, 2002). MAPK phosphatases (MKP) dephosphorylate and thus return MAPKs to an inactive state.

In multicellular organisms, there are three subfamilies of MAPKs identified so far. The first subfamily is extracellular signal–regulated kinases (ERKs) which have several isoforms (Zhou et al., 1995). ERK1 and ERK2 are the best characterized. The second subfamily is c-Jun $NH_2$-terminal kinase (JNKs). There are three major JNKs, JNK 1-3, classified according to three jnk genes. For p38, four isoforms (p38α, β, γ and δ) have been found.

MAPKs can be activated by a wide variety of stimuli, but in general, ERK1 and ERK2 are preferentially activated in response to growth factors. In contrast, JNK and p38 kinases are more responsive to stress stimuli ranging from osmotic shock and ionizing radiation to cytokine stimulation (Pearson et al., 2001). It is worth noting that there is increasing evidence of ERK activation by stress stimuli, for example, oxidative stress and hyperosmotic stress (Wang et al., 1998).

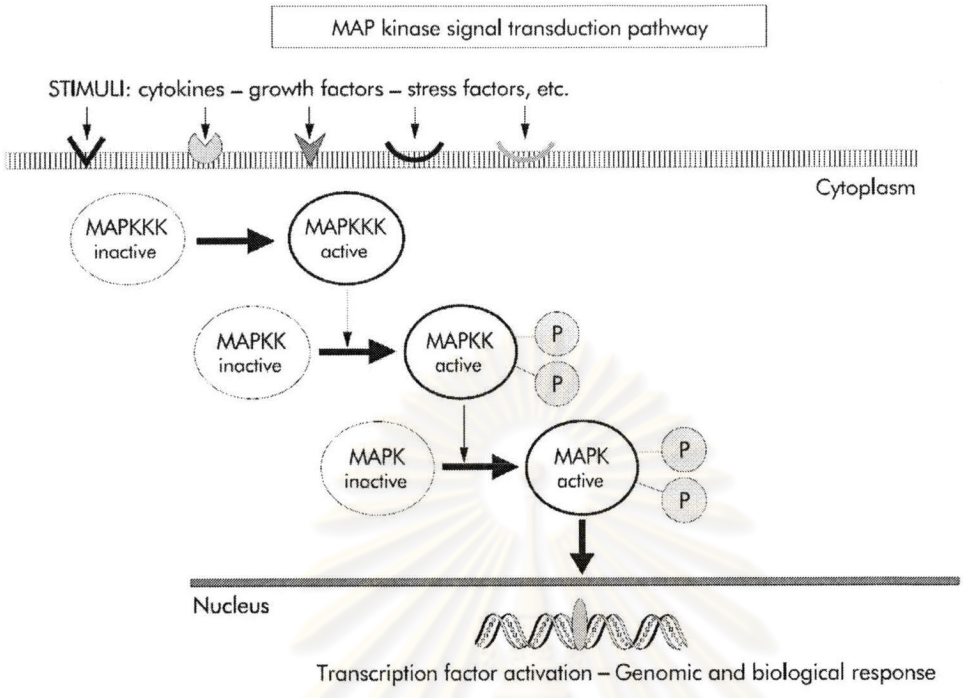


**Figure 1** Activation of MAPK cascades. Different extracellular stimuli can activate the family of mitogen activated protein (MAP) kinases after receptor-ligand interactions. Members of this family activate each other by adding phosphate groups to serine/threonine amino acids (Hommes et al., 2003).

## The ERK pathway

The ERK signalling pathway is the first MAPK subfamily characterized, ERK is a vital mediator of a number of cellular events ranging from growth, proliferation to survival. Two ERK isoforms, ERK1 and ERK2, are the most characterized and are often referred to as p44 and p42 MAPKs, respectively, according to their molecular weights (Boulton et al., 1991). In this cascade, MEK1 and MEK2 function as upstream MAPKKs and Raf proteins as MAPKKKs (figure 2). Cell surface receptors such as tyrosine kinase receptor and G protein-coupled receptors transmit activating signals to the Raf/MEK/ERK cascade via the small GTP-binding protein Ras (Wood et al., 1992; Campbell et al., 1998). Activation of membrane-associated Ras is

achieved through recruitment of SOS, a Ras-activating guanine nucleotide exchange factor. SOS stimulates Ras to change GDP to GTP, allowing it to interact with a wide range of downstream effector proteins, including Raf (Geyer et al., 1997). The exact mechanism of Raf activation is still elusive but is known to require Ras binding (Chong et al., 2003). Activated Raf binds to and phosphorylates MEK1/2, which in turn phosphorylate ERK1/2 within a conserved Thr-Glu-Tyr (TEY) motif in their activation loop. Duration of ERK1/2 activation depends on the removal of one or both phosphates by specific phosphatases (Todd et al., 1999).

Upon stimulation, a significant population of ERK1/2 accumulates in the nucleus (Chen et al., 1992; Gonzalez et al., 1993; Lenormand et al., 1993). While the mechanisms involved in the nuclear accumulation of ERK1/2 remain elusive, nuclear retention, dimerization, phosphorylation, and release from cytoplasmic anchors have been shown to play a role (Pouyssegur et al., 2002). Then, the activated ERKs translocate to the nucleus and phosphorylate different transcription factors, such as Elk-1, c-Myc

**The JNK pathway**

The JNK signalling pathway is a stress-activated pathway that is involved in the regulation of cell proliferation and apoptosis. JNK protein kinases are encoded by three genes jnk1, jnk2 and jnk3, whose mRNAs are alternatively spliced to form 10 JNK isoforms (Gupta et al., 1996). These isoforms can be grouped into JNK1, JNK2, and JNK3 according to corresponding genes. JNK 1 and 2 are ubiquitously expressed, but JNK3 is present primarily in the brain. The JNKs are strongly activated in response to cytokines, UV irradiation, growth factor deprivation, DNA-damaging agents, and, to a lesser extent, some G protein-coupled receptors, serum, and growth factors (Kyriakis and Avruch, 2001).

Like ERK and p38, JNK activation requires dual phosphorylation on tyrosine and threonine residues within a conserved Thr-Pro-Tyr (TPY) motif. The MAPKKs that catalyze this reaction are known as MEK4 and MEK7, which are in turn phosphorylated and activated by several MAPKKKs, including MEKK1-4, TAO1-2, and ASK1-2 (Kyriakis and Avruch, 2001). The major substrate of JNKs is the transcription factor c-Jun. Phosphorylation of c-Jun on Ser63 and Ser73 by JNKs leads to increased c-Jun-dependent transcription (Weston and Davis, 2002). Other transcription factors that have been shown to be phosphorylated by the JNKs are ATF-2, NF-ATc1and STAT3 (Chen et al., 2001; Kyriakis and Avruch, 2001).

## The p38 pathway

The mammalian p38 MAPK subfamily is activated by cellular stresses including UV irradiation, heat shock, high osmotic stress, lipopolysaccharide, protein synthesis inhibitors, proinflammatory cytokines (such as IL-1 and TNF-α) and certain mitogens. To date, four p38 isoforms (p38α, p38β, p38γ and p38δ) have been identified sharing about 60% homology and two isoforms (p38α, p38β) are ubiquitously expressed (Ono and Han, 2000). p38γ is predominantly expressed in skeletal muscle, whereas p38δ gene expression is found in lung, kidney, testis, pancreas, and small intestine. All these isoforms of p38 can be phosphorylated by the MAPK kinase MKK6 (SKK3). MKK3 can activate p38α, p38β and p38γ, whereas MKK4 can activate only p38δ. These MKKs can be activated by MEKK1-4, MLK2-3, DLK, ASK1, Tpl2 (also termed Cot), and Tak1. Activation of the p38 isoforms is a result of MEK3/6-catalyzed phosphorylation of a conserved Thr-Gly-Tyr (TGY) motif in their activation loop.

p38 MAPK appears to play a major role in apoptosis, differentiation, survival, proliferation, development and inflammation. A large body of evidence indicates that p38 activity is critical for normal immune and

inflammatory responses. p38 is activated in macrophages, neutrophils, and T cells by numerous extracellular mediators of inflammation, including chemoattractants, cytokines, chemokines, and bacterial lipopolysaccharide (Ono and Han, 2000). Apart from the inflammatory mediators, p38 MAPKs are activated by many other stimuli, including hormones, ligands for G protein–coupled receptors, and stresses such as osmotic shock and heat shock.

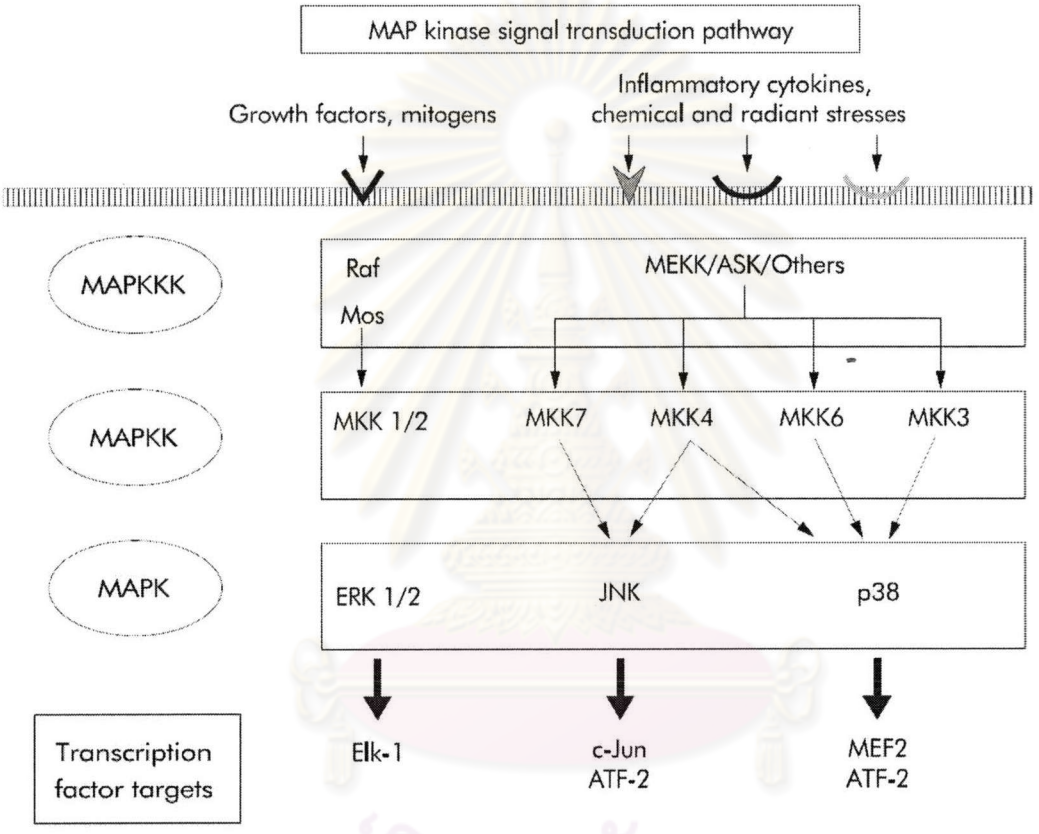


**Figure 2** Activation of the three main MAPKs. Result of phosphorylation of various MAPKKs and MAPKKKs is activation of ERK, JNK, and p38, which have different transcription targets (Hommes et al., 2003).

## 2.2 Roles of MAPKs in the nervous system

MAPKs are known to play a role in cell proliferation and differentiation, stress responses, immune responses as well as cell survival (Chang and Karin, 2001). The roles of MAPKs on cell survival are well understood. In general, activation of ERK has been linked to cell survival while JNK and p38 are associated with induction of apoptosis; however, the actual role of each MAPK pathway is highly dependent on stimuli and cell type. For example, cell survival promoted by ERK has been reported in keratinocytes and HeLa cells (Wang et al., 1998; Peus et al., 1999). By contrast, ERK-induced apoptosis has been shown in astrocytes (Ramesh and Philipp, 2005). Therefore, the roles of MAPKs in neurons and glial cells will be focused due to the pertinence to the context of this thesis.

The ERK pathway is involved in neurotrophin-dependent survival and differentiation of developing peripheral neurons (Obata and Noguchi, 2004). In retinal ganglion cells, an anti-apoptotic role of ERK by increasing Bcl-2 and decreasing Bax has been demonstrated (Rios-Munoz et al., 2005). Nevertheless, in other neuronal cell types, activated ERK can induce apoptosis after $K^+$ deprivation by a pathway distinct from the caspase cascade (Subramaniam et al., 2004). Therefore, the role of ERK in neuronal survival may depend on cell types. In the PNS, ERK is present in dorsal root ganglion (DRG) neurons, satellite cells as well as Schwann cells (Averill et al., 2001) and appears to play a role in neurite outgrowth responses to neurotrophin NGF and IGF-1 (Kimpinski and Mearow, 2001). The role of ERK in PNS glial cells is still unknown but one study has reported that Ras/Raf/ERK signalling can drive the dedifferentiation of Schwann cells, similar to that occur following nerve injury (Harrisingh et al., 2004).

In vitro, activation of JNK leading to neuronal death is well recognized. For example, following NGF deprivation, JNK and its main transcription factor

c-Jun are activated in PC12 cells and cultured cerebellar granule neurons leading to cell death (Lelkes et al., 2001; Cao et al., 2004). In animal model, JNK activation in DRG which contributes to pain hypersensitivity has been reported (Doya et al., 2005). Nevertheless, the effect of JNK activation in the PNS is largely unknown. JNK also phosphorylates neuronal microtubule regulator that contributes to the dynamic assembly and disassembly of microtubules (Neidhart et al., 2001). Considering different isoforms, JNK 1 and 2 are widely expressed and JNK3 isoform is predominantly present in the nervous system. JNK3-deficient sympathetic neurons are resistant to NGF-deprivation-induced apoptosis (Bruckner et al., 2001), supporting the hypothesis of JNK involvement in neuronal apoptosis. However, the precise role of each JNK isoform in the nervous system remains to be classified.

Evidence suggesting that p38 activation plays a role in neuronal cell apoptosis has been reported. For example, inhibition of p38 activation by Bcl-2 can prevent A beta-induced PC12 apoptosis (Song et al., 2004), as well as nitric oxide-induced neural progenitor cell death (Cheng et al., 2001). In vivo, spinal cord injury has been reported to cause p38 and caspase-3 activation while SB203580, an inhibitor of p38, significantly reduced the number of apoptotic cells in the injured spinal cord (Wang et al., 2005). The role of p38 pathway in peripheral neurons and associated glial cells is largely unknown and remains to be elucidated. However, at least, the deleterious role of p38 in the PNS has been demonstrated. p38 is activated in neurons cultured in high-glucose condition and in DRG from diabetic animals, and inhibition of p38 is associated with reduced cell death and improved electrophysiological abnormalities (Purves et al., 2001; Price et al., 2004). Although p38 is widely expressed in many tissues, p38$\alpha$ and $\beta$ are major isoforms present in the brain. Because of this, the pro-apoptotic role of p38 observed in neurons could be driven mainly by these two isoforms (Harper and Lograsso, 2001).

In summary, MAPKs are implicated in neuronal differentiation, stress responses and survival. In the mammalian nervous system, the role of ERK in neuronal cell death is still controversial and appears to be dependent on cell type, while the implication of JNK and p38 in neuronal death is more consistently evident. Because most evidence comes from the studies with the central nervous system (CNS) neurons and glial cells, future studies should point to clarify the roles of MAPKs in not only CNS but also PNS neurons and associated glial cells and not only in cell survival but also other aspects.

## 2.3 Paclitaxel-induced MAPKs phosphorylation in neurons

Although paclitaxel (Taxol®) is widely used and is known to activate a number of signal transduction pathways that induce programmed cell death (apoptosis) in a variety of cell types, the precise underlying mechanisms are poorly understood.

ERK has been proposed as pro-survival signal responding to paclitaxel treatment in many cell types. However, in neuroblastoma SK-N-SH cells, Guise and colleaques (Guise et al., 2001) have shown that a sustained activation of ERK induced both apoptosis and phosphorylation of tau, a neuronal microtubule associated protein, which plays a significant role in the disruption of microtubules leading to apoptosis. These events were reduced by inhibition of ERK using PD98059 or antisense strategy, indicating a link between ERK activation, tau phosphorylation and apoptosis. The role of paclitaxel-induced ERK activation in neurons may be different from other cell types. However, more studies are needed to clarify this aspect.

Waetzig and Herdegen (Waetzig and Herdegen, 2003) have shown that the treatment of PC12 cells with paclitaxel activated all JNK isoforms while protein expression did not change. In addition, both ATF-2 and c-jun, the well known substrates of JNK, were also phosphorylated and apoptosis was

observed. The pro-apoptotic role of JNK was confirmed by transfection of PC12 cells with JNK, and found that the transfected cells had increased phosphorylation of ATF-2 and c-jun, and enhanced cell death after paclitaxel treatment. In addition, JNK-induced apoptosis mediated through Bcl-2 and c-Raf phosphorylation has been demonstrated in SH-HY5Y cells (Nicolini et al., 2003).

Arvidsson (Arvidsson et al., 2001) has demonstrated a pro-apoptotic role of p38 in paclitaxel treated neuroblastoma cell lines. Apoptosis signal-regulating kinase 1 (ASK1), a MAPK kinase kinase which is typically activated by TNF-α and in turn activates JNK and p38, was activated and caused cell death mainly via the p38 pathway. Moreover, Chen and Cobb (Chen and Cobb, 2001) have also shown another mechanism of p38-induced apoptosis in paclitaxel-treated neuronal cells. TAO2, a protein kinase that phosphorylated MEK3 and MEK6, upstream regulators of p38, is activated by paclitaxel followed by p38 phosphorylation. However, the authors did not study the survival rate of cells.

In conclusion, activation of MAPKs responding to paclitaxel in neuronal cells has been demonstrated and the pro-apoptotic role of this MAPK activation leading to neuronal cell death has been proposed. However, it should be kept in mind that most evidence comes from in *vitro* experiments using tumor cells. As a result, more in *vivo* studies are needed to elucidate the effect of paclitaxel-induced MAPK activation in the nervous tissue. Furthermore, possible involvement of other signaling pathways and crosstalk among these pathways in the apoptosis in response to the microtubule-targeting agents should be examined. Better understanding of the signal transduction pathways activated by paclitaxel will be useful in both prevention and treatment of paclitaxel-induced neuropathy.