The importance of intracellular trafficking in cancer cells and in treatment

PhD thesis

Dr. Edina Komlódi-Pásztor

Semmelweis University

PhD School, Molecular Medicine

Adviser: Dr. András Váradi, Ph.D., DSc

Opponents: Dr. Krisztina Káldi, Ph.D.
Dr. László Bálint, MD-Ph.D.

Comprehensive Exam Committee:

President: Dr. Mária Sasvári, professor

Members: Dr. Katalin Jemnitz, Ph.D.
Dr. Miklós Csala, MD-Ph.D.

The PhD work was done in the laboratory of Dr. Tito Fojo, MD-Ph.D. at the National Institutes of Health, National Cancer Institute, Bethesda, MD, USA

Bethesda, Maryland, United States

2010.

1. Table of content

2. Abbreviations

2-ME	2-methoxyestradiol
^{3H}TdR	^{3H}thymidine
BR	basic region
C	cytoplasmic
CHX	cycloheximide
CML	chronic myeloid leukemia
CP	cisplatin
DBD	DNA-binding domain
ER	endoplasmic reticulum
FBS	fetal bovine serum
H3	histone 3
HR	hematological remission
KSP	kinesin spindle protein
LI	Labeling index
LMB	leptomycin B
MR	minor response
MT	microtubules
MTOC	microtubule organizing center
N	nuclear
NES	nuclear export signal
NLS	nuclear localization signal
OX	oxaliplatin
PD	progressive disease
PFS	progression-free survival
PLK	polo-like kinases
PR	partial response
PRR	proline-rich region
PSD	prolonged stable disease
RR	relative resistance
RT	room temperature
TAD	transactivation domain
TMD	tetramerization domain
TR	transient response

3. Introduction

3.1. Brief history of p53 protein research

During its 29-year history, p53 has remained in the spotlight of cancer research and accomplished a unique scientific journey in molecular oncology. The p53 protein was identified independently in the laboratories of Arnold Levine (Linzer & Levine, 1979), David Lane (Lane & Crawford, 1979) and William Old (DeLeo et al., 1979) in 1979. Its gene, called TP53, was first cloned from mice by Peter Chumakov in 1982 and independently by Oren in 1983 (Oren & Levine, 1983). In the following year, Maltzman and Czyzyk demonstrated that the level of p53 is increased following DNA damage induced by UV irradiation (Maltzman & Czyzyk, 1984). In the same year, the laboratory of Rotter identified p53 gene alterations in virus-induced leukemias (Wolf et al., 1984). For a decade, it was presumed that p53 acts as an oncogene, until the work on rodents by the groups of Levine and Oren, and the work on colorectal carcinomas by Bert Vogelstein changed this paradigm by demonstrating the tumor suppressor function of p53. The confusion resulted from the fact that originally a mutant p53 sequence was considered as the wild type protein. Since then, p53 protein has been often referred to as "the guardian of the genome" because its proper activity is crucial for normal cell function (Lane, 1992). Although not required for normal cell growth and development, p53 plays a significant role in DNA repair, angiogenesis, cell cycle arrest, and apoptosis. In 1992, the generation of p53 null mice and the demonstration of their increased susceptibility to tumorigenesis added further support to the paradigm of p53 as a tumor suppressor. Because it was in the center of research, p53 was selected the "Molecule of the Year 1993" by Science (Beijnen, 1994). The next important milestone in the history of p53 research was the description of the p53 crystal structure. The structure of the p53 core domain bound to DNA was defined in 1994 by the group of Pavletich (Cho et al., 1994). Three decades of intensive research has resulted in an impressive amount of information about p53. The growing knowledge of p53 originating from laboratory studies has been translated into a greater understanding of its clinical relevance. In the future, the development of treatment regimens guided by the tumor's p53 status will hopefully lead to more effective cancer management and better outcomes.

3.2. The structure of p53 protein

Human p53 is a 393 amino acid protein. It is comprised of three domains, defined as independently folding units, which are connected to each other by proline-rich linker regions (Table 1, Figure 1). The N-terminus domain is referred to as the transactivation domain (residues 1-63), and is followed by the first proline-rich region (residues 64-92). The DNA-binding domain (residues 100-293), the largest and the most frequently mutated unit, is situated in the middle section of the protein and is flanked by proline-rich regions at each side. The second proline-rich domain (residues 294-323) links the DNA-binding domain to the tetramerization domain (residues 324-355). Finally, the C-terminus of the protein is called the basic region (residues 356-393). In the paragraphs that follow, I discuss the p53 structure in greater detail.

Residues	Name
1-63 residues	Transactivation Domain
64-92 residues	Proline-rich Region
100-293 residues	DNA Binding Domain
294-323 residues	Proline-rich Region
305-322 residues	Nuclear Localization Signal I.
310-360 residues	Tetramerization Domain
311-393 residues	DNA Damage Recognition Region
319-360 residues	Oligomerization Region
326-355 residues	Cytoplasmic Sequestration Region
340-351 residues	Nuclear Export Signal
369-375 residues	Nuclear Localization Signal II.
379-384 residues	Nuclear Localization Signal III.
356-393 residues	Basic Region

Table 1. Table of the domains and regions of the human p53 protein

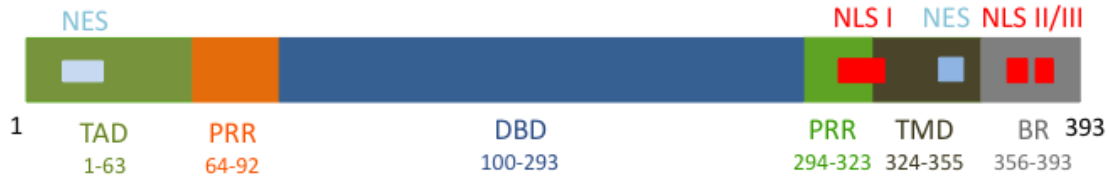


Figure 1. Schematic structure of the human p53 protein
TAD: transactivation domain, PRR: proline-rich region, DBD: DNA-binding domain, TMD: tetramerization domain, BR: basic region, NES: nuclear export signal, NLS: nuclear localization signal

The transactivation domain, localizing to the N-terminus of the protein, serves at least two roles: it activates sequence-specific transcription of genes and regulates p53 stability and function (Shieh et al., 1997). The term "transactivation domain" is inaccurate since this region itself is not an independent folding unit. However, since this name has been used in the literature extensively, it is unlikely to change. The transactivation domain is capable of interacting with several regulatory proteins (p300, CBP and Hdm2) that alter the stability and the transcription activity of p53 by post-translational modifications (Xu, 2003). Phosphorylation of the N-terminus of the protein reduces the affinity of p53 for Hdm2 (the negative regulator of p53) and results in increased association with p300/CBP. Moreover, this domain physically interacts with Hdm2. Thus, Hdm2 leads to non-functional p53 in at least two ways: 1) by covering its transactivation domain and 2) by initiating post-translation modifications on the protein that results in proteasomal degradation of p53.

The first proline-rich region, often called the polyproline region, has ten prolines, arranged in five copies of the motif "PXXP". It has been associated with regulation of p53-induced apoptosis (Walker & Levine, 1996). It may have a role in the proper folding of the protein.

The DNA binding domain, often referred to as the "core domain", is probably the most examined part of the protein. The reason why this region has stood in the center of interest is that this is the most frequently mutated domain of p53 in human

cancers (Hainaut & Hollstein, 2000). This domain is responsible for the binding of p53 protein to DNA, the first step in the complex process of gene transcription. Because of its important role in normal cell function, it is not surprising that this region contains the most evolutionally conserved sequences among different species and among the different members of the p53 family (p63, p73). The consensus p53 binding sequence in DNA is as follows: RRRCWWGYYY where R stands for a purine, Y for a pyrimidine and W is an adenine or a thymine. This 20-nucleotide binding site in the double helix is recognized by the p53 tetramer. Certain amino acids have special importance in this domain of p53: residues 175, 249 and 282 stabilize the native structure; whereas, residues 248 and 273 contact the DNA directly.

The putative function of the second proline-rich domain is to promote the proper folding of the DNA bound p53 tetramer by accommodating the different symmetry of the DNA binding and tetramerization domains in the monomer. This region contains seven prolines.

The tetramerization domain is responsible for the arrangement of the active form of p53, the p53 tetramer. The complex is actually a dimer of two dimers and within one dimer the N-terminus of one monomer is located close to the C-terminus of the associated monomer. This dimer conformation was described as “twins facing each other and holding hands” by the group of Orlova (Okorokov et al., 2006). The nuclear export signal and part of the nuclear localization signal I reside in this region.

The basic region at the C-terminus of the protein is also in the spotlight due to its mysterious and contradicting roles in p53 function. This region has been shown to interact with DNA in a sequence nonspecific manner (Foord et al., 1991). In addition to its role in non-specific DNA binding, this region also determines the function and stability of p53 based on post-translational modifications, such as sumoylation, phosphorylation, acetylation and glycosylation (Appella & Anderson, 2001). The exact role of these modifications is under intense investigation. This region also includes two minor nuclear localization signals, NLS II and NLS III.

3.3. p53 mutations

Two types of p53 mutation can be distinguished: somatic and germline mutations. In my work, I will focus on somatic p53 mutations and I will not discuss germline mutations, such as those present in patients with the Li-Fraumeni syndrome.

Mutations of TP53 are the most frequently documented abnormalities in human cancer (Soussi & Beroud, 2001). It is well known that more than 50% of a diverse group of cancers harbor mutant p53 proteins. The majority (90-95%) of these p53 mutations occur in the DNA binding domain of the protein and these alterations lead to functional inactivation of p53 (Figure 2).

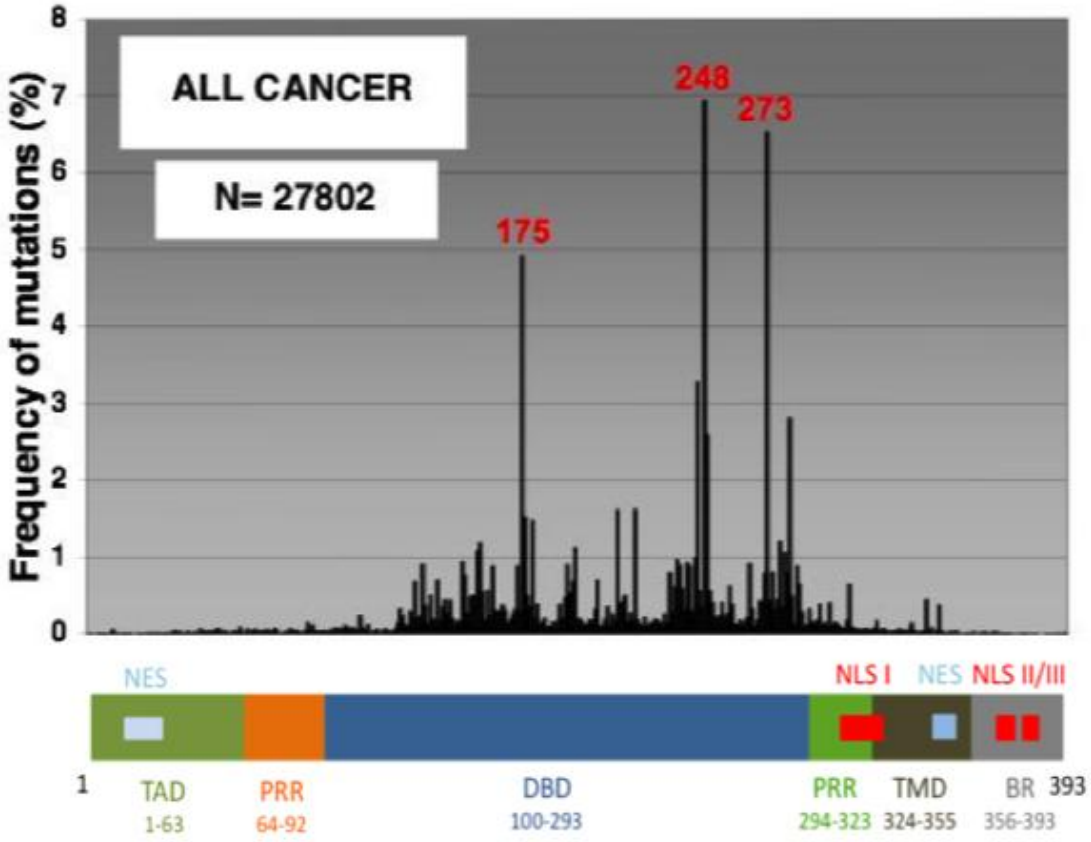


Figure 2. Schematic structure of p53 and the frequency of p53 mutations
Adopted from http://p53.free.fr/Database/p53_cancer/all_cancer.html

Soussi's laboratory collected almost 28,000 p53 mutations and established the UMD-p53 database, the most complete database on p53 (2010) (http://p53.free.fr). According to this database the most common mutation type is the missense mutation; it represents more than 70% of the alterations (Figure 3). Among these, the most common base substitution is the GC → AT transition. This transition commonly occurs at CpG dinucleotides, which are frequently methylated. On the other hand, the least frequent type of mutation, a gene deletion, is present in only in 0.1% of cases.

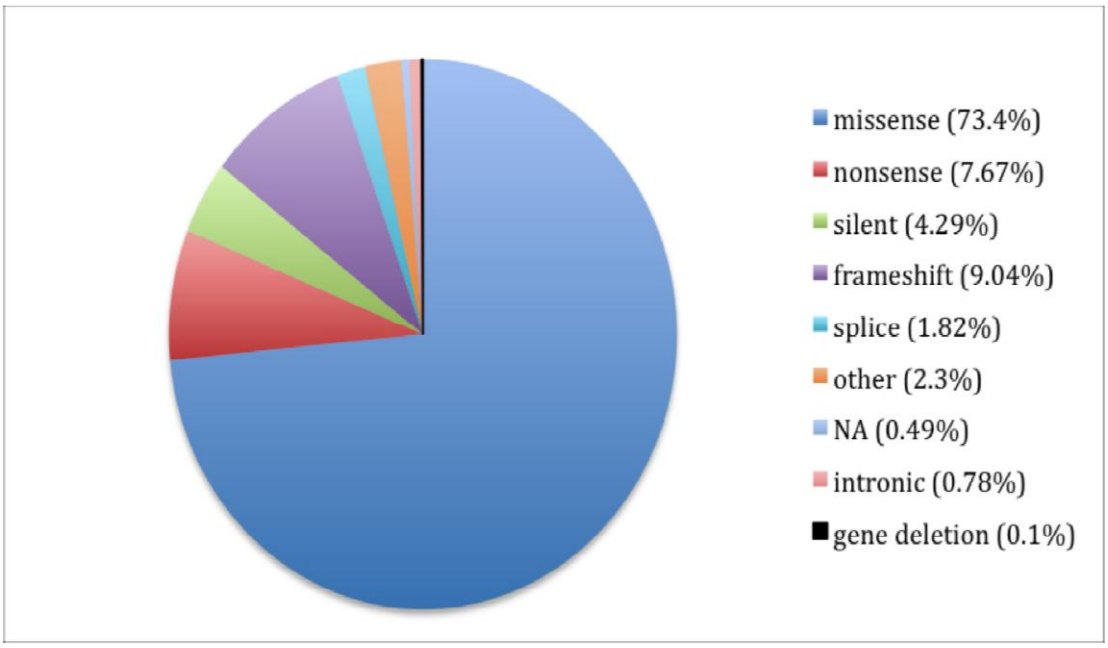


Figure 3. Types and distribution of mutations found in p53 protein
Adapted from http://p53.free.fr

Mutations in the DNA binding domain are not randomly distributed, but instead are found more frequently at specific residues. Most missense mutations target codons that are evolutionarily conserved. Crystallographic structure analysis, has shown that the most frequently mutated residues of p53 have a significant role in binding the protein directly to the DNA. Mutations affecting residues 175, 248, 249, 273 and 282 result in a p53 protein that lacks the ability to bind DNA and initiate sequence-specific DNA transcription either as a result of awkward DNA binding or because of the instability of the native structure of p53. Very few mutations in human tumors (around

5%) affect the N- or the C-termini of the protein, and these are mainly nonsense and frame-shift mutations.

Primary sequence mutations are not the only way in which the function of p53 is eliminated. Neutralization by viral products, upstream regulator- or downstream effector signal alterations, and cytoplasmic sequestration are also common forms of p53 inactivation. When the function of p53 is lost, cells become vulnerable and can accumulate more DNA damage, such as mutations, gene amplification and chromosomal rearrangements. From a clinical point of view, functionally inactivated p53 results in resistance to chemo- and radiotherapy due to loss of apoptotic competence.

3.4. Microtubules and the intracellular trafficking of p53

The p53 protein, like many other molecules, moves from sites of synthesis and storage to sites of function. Intracellular trafficking occurs on actin filaments and on microtubules. While actin filaments promote short distance movements mainly near the outer membrane, microtubules are responsible for long distance bidirectional movement of a variety of cargos (Ross et al., 2008). Bidirectional transport (anterograde and retrograde) occurs because the ends of cellular microtubules are not identical. The polarity of microtubules is the consequence of the repeating α/β-tubulin heterodimers that bind head to tail into protofilaments. The parallel association of protofilaments forms the microtubule wall (Figure 4) (Akhmanova & Steinmetz, 2008). In interphase cells, cellular arrays of microtubules are organized such that all the component microtubules have the same end near the nucleus (referred to as the “minus” end), and the opposite end (“plus” end) at the periphery, near the outer membrane (Gross, 2004) (Figure 5). During mitosis the “minus” end attaches to the centrosome, and the “plus” end attaches to the kinetochore (Figure 6). The “plus” end of the microtubules is the rapidly growing end that is stabilized by a GTP-cap, a cluster of α/β dimmers with bound GTP. The loss of this cap results in the rapid degradation of the microtubules. Microtubules are highly dynamic, they are able to polymerize or depolymerize rapidly. A class of chemotherapeutic agents has been developed over the last quarter century that interferes with the assembly of microtubules.

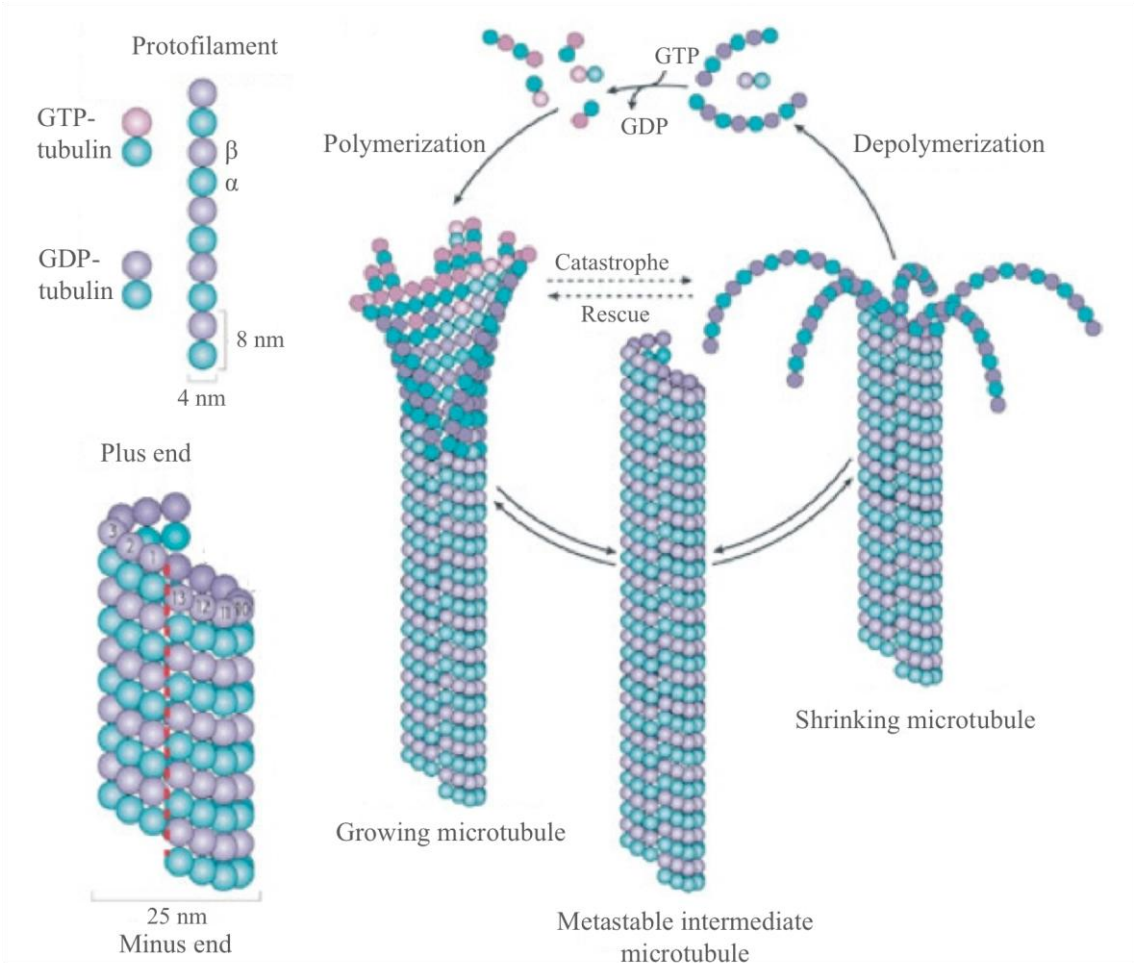


Figure 4. The structure and assembly of microtubules
Akhmanova & Steinmetz. Nature Reviews Molecular Cell Biology 9, 309-322 (2008)

Because of their critical role in the organization of microtubules, centrioles are often referred to as the microtubule organizing centers (MTOC). They are composed of two orthogonally arranged centrioles surrounded by a pericentriolic matrix composed of proteins responsible for microtubules nucleation and assembly (Figure 6). A centriole comprises nine sets of triple microtubules. Centrosomes duplicate during interphase but stay close together and adjacent to the nucleus. As cell division begins, the two centrioles (MTOC) move to opposite poles of the cell and microtubules radiates from them. This star-shape arrangement is often referred to as asters. For separating the sister chromatids, microtubules form spindle fibers that connect to the kinetochore and generate force for the movement (Figure 5).

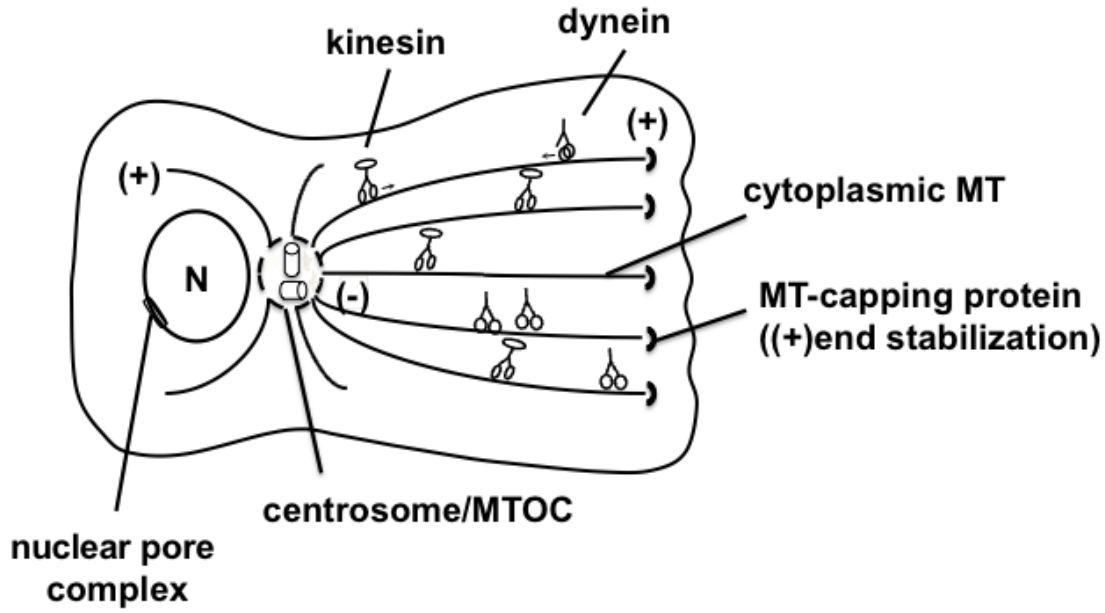


Figure 5. Interphase cell – organization of microtubules

Adapted from http://www.colorado.edu/MCDB/MCDB1150/ohd/mtoc.jpg

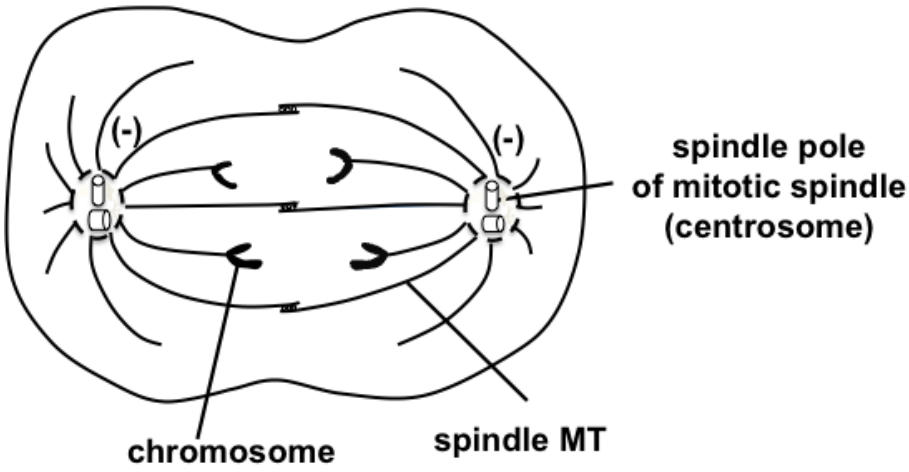


Figure 6. Mitotic cell – two microtubules organizing centers

Adapted from http://www.colorado.edu/MCDB/MCDB1150/ohd/mtoc.jpg

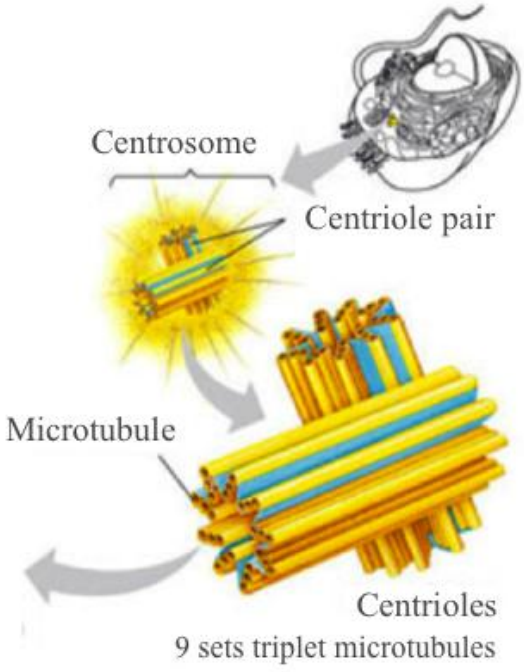


Figure 7. Schematic figure of centrosome. Adapted from: http://scienceblogs.com/transcript/2006/08/new_evidence_for_endosymbiotic.php

Microtubule motor proteins interact with tubulin in order to move cargo on microtubules. These mechanochemical enzymes hydrolyse ATP to generate forces to carry the cargo. Two classes of motor proteins are recognized: one for retrograde movement of cargo (dynein, the negative end motor protein), and one for anterograde movement (kinesin family, the plus end motor proteins) (Figure 5). Although these two types of motor proteins play similar roles in intracellular trafficking, they are fundamentally different from each other. Kinesin has a relatively simple structure; it contains two heavy and two light chains. It does not require linker proteins since kinesin specifically recognizes its cargos, such as secretory vesicles, ER membranes, pigment granules, and lysosomes. Dynein is much larger and more complex than kinesin. It contains two or three heavy chains, and several intermediate and light chains; and as a result, its molecular weight is over a million kDa. Dynein is not able to directly bind its cargo (e.g. proteins, vesicules, chromosomes) but requires a linker protein such as dynactin.

The level and localization of p53 is tightly regulated (O'Brate & Giannakakou, 2003). Under normal conditions, p53 has a very short half-life (6-20 minutes) and the protein is maintained at low steady state levels (Reich et al., 1983). Cellular stresses, including DNA damage, hypoxia, ribonucleoside triphosphate depletion, mitotic spindle damage, NO signaling and oncogene activation, stabilize p53 by post-transcriptional modifications that in turn lead to increased intracellular levels of p53 (Graeber *et al.*, 1994; Linke *et al.*, 1996; Lowe & Ruley, 1993; Maltzman & Czyzyk, 1984; Meek, 2000; Wang *et al.*, 2002). The stabilization of p53 promotes the formation of a tetramer, which is the active form of p53. The active tetramer binds to dynein in a microtubule independent way, then the p53-dynein complex binds to the microtubules in order to traffic to the perinuclear region (Giannakakou et al., 2000). Once at the perinuclear region, p53 binds the importin heterocomplex in order to translocate to the nucleus (Figure 8). Studies have shown that mutations altering the p53 tetramerization domain interfere with the protein's intracellular localization (Trostel et al., 2006). Moreover, disruption of the microtubule network by microtubule targeting agents, such as taxol or vincristine, leads to p53 cytoplasmic accumulation (Figure 9) (Giannakakou *et al.*, 2000).

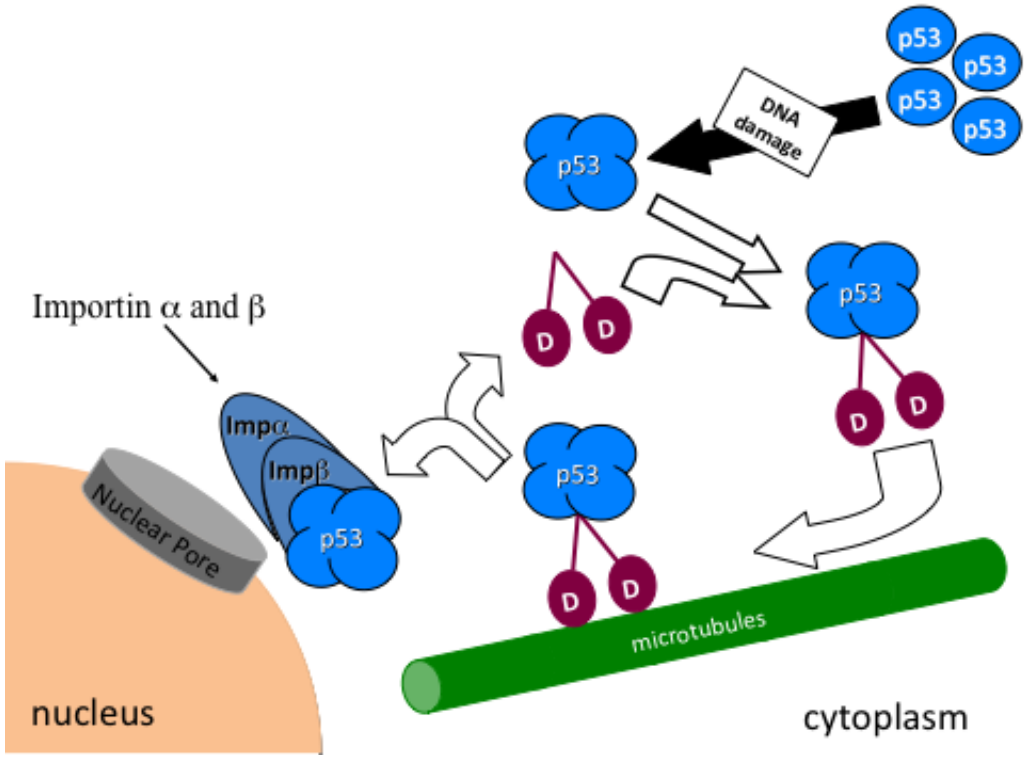


Figure 8. Intracellular trafficking of p53

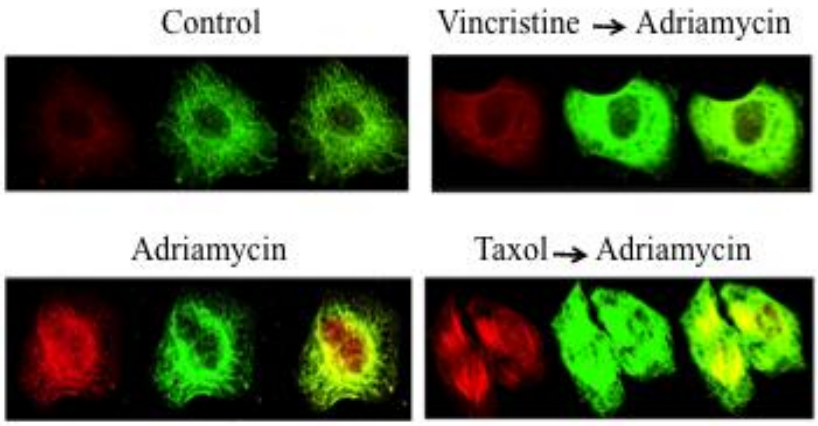


Figure 9. Adriamycin leads to p53 nuclear accumulation that can be prevented by pretreatment with a microtubule-targeting agent (red: p53, green: tubulin, yellow: merged) (Giannakakou *et al.*, 2000)

It has been previously believed that p53 has three putative nuclear localization signals (NLS). Each consists of a basic amino acid core surrounded by alpha-helix-breaking amino acids (Shaulsky et al., 1990). Studies have reported that nuclear translocation of p53 is mediated principally by NLS I, with NLS II and NLS III serving in an ancillary capacity (Liang & Clarke, 1999; Shaulsky *et al.*, 1990) (Figure 10, Table 2). For nuclear translocation, the nuclear localization signals of p53 interact with a carrier heterocomplex, importin-α/β. Importin-β establishes the connection between the p53-bound heterocomplex and the nuclear pore complex, while importin-α carries the protein during the translocation (Gorlich et al., 1995; Lange et al., 2007). Following nuclear translocation, p53 dissociates from importin-α releaasing in side the nucleus and allowing it to bind DNA at p53 responsive elements in order to initiate gene transcription of a myriad of genes, including Hdm2, its own negative regulator. X-ray crystallographic analysis has shown that residues 25-109 of Hdm2 form a hydrophobic pocket in which the N-terminus of p53 fits tightly, much like a key and a lock. In this interaction, Hdm2 acts as an E-3 ubiquitin ligase and ubiquitinates the C-terminus of p53. Hdm2 mediated ubiquitination promotes p53 nuclear export by allowing the interaction between the nuclear export signal of a p53 monomer and Exportin 1, a nucleocytoplasmic carrier. In the cytoplasm, p53 undergoes degradation by the 26S proteasome.

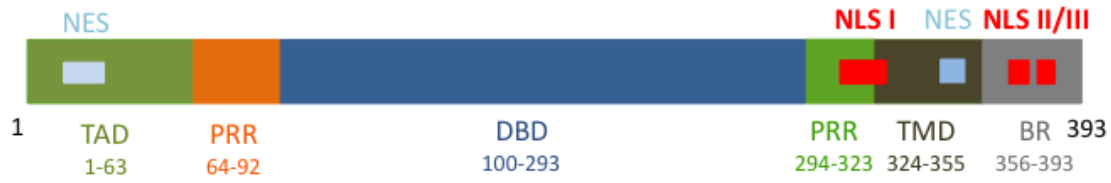


Figure 10. Schematic structure of p53 – NLSI-III are found at the C-terminal of the protein

Residues	**Name**
305-322 residues	Nuclear Localization Signal I.
369-375 residues	Nuclear Localization Signal II.
379-384 residues	Nuclear Localization Signal III.

Table 2. Table of regions of nuclear localization signals

A tumor with wild-type p53 and hyperactive Hdm2 is not able to increase p53 protein levels due to its augmented degradation, and presents a p53-null phenotype. On the other hand, Mdm2 knock out mice display early embryonic lethality because of hyperactive p53-induced apoptosis. Deleting both genes (p53 and Hdm2) rescues embryonic lethality confirming the strong co-operation between the two molecules. Interestingly, the level of Hdm2 defines the location of p53 degradation. Low levels of Hdm2 lead to mono-ubiquitination of p53 and nuclear exclusion. Apparently, the cytoplasmic proteasome is responsible for the degradation of this protein. However, when Hmd2 levels are high, p53 becomes poly-ubiquitinated and its rapid proteasomal degradation takes place in the nucleus (Li et al., 2003) The energy for nuclear import and export is provided by the small Ras-family GTPase, Ran (Quimby & Dasso, 2003).

Some inflammatory breast cancers, melanoma malignum, colon cancers as well as undifferentiated neuroblastomas present with a loss of p53 activity secondary to cytoplasmic sequestration of the wild-type protein (Bosari et al., 1995; Lilling et al., 2002; Moll et al., 1995; Weiss et al., 1995). In these cases, the potentially functional

wild-type p53 is sequestered in the cytoplasm, and cannot promote tumor growth. Reports suggest cytoplasmic p53 portends a poor survival in patients with colon cancer (Bosari et al., 1994; Sun et al., 1992). Several descriptive studies in the literature have failed to discriminate among the possible explanations of p53 cytoplasmic accumulation (Bosari *et al.*, 1995; Imamura *et al.*, 1993; Weiss *et al.*, 1995). Others reported p53 accumulation in the cytoplasm either as a result of insufficient nuclear translocation or because of augmented nuclear export (Kim et al., 2000; Stommel et al., 1999). The list of putative contributing factors for p53 cytoplasmic sequestration are long, and include a truncated form of importin alpha (Kim *et al.*, 2000), increased levels of intermediate filaments (Sembritzki et al., 2002), cytomegalovirus infection (Utama et al., 2006), endoplasmic reticulum stress (Qu et al., 2004), amplification of unknown genes inducing protein complex formation with p53 (Ottaggio et al., 2000) and aberrant hyperubiquitination (Becker et al., 2007), among others.

The clinical consequence of these phenomena is that tumor types with non-functional p53 (either by mutation in the wild-type sequence or by inappropriate subcellular localization) are less responsive to chemotherapy and radiotherapy because of the inability of p53 to initiate apoptosis (Weinstein et al., 1997). It is not surprising that the regulation of p53 protein localization is tightly controlled to ensure the proper nuclear homing of p53 following different cellular stress.

3.5. Nuclear localization signals of p53

For normal cell function, proteins shuttle between the cytoplasm and the nucleus. Small proteins and ions can enter the nucleus by passive diffusion. RNAs and proteins larger than 40 kDa need special transporters, called karyopherins, and energy in order to translocate to the nucleus. Proteins, which need assistance in translocation harbor specific sequences, called nuclear localization signals (NLS), in their structures. Nuclear localization signal I (NLS-I) of p53 is highly conserved across different species and shares perfect homology with a consensus nuclear localization signal sequence. Nuclear localization signals II and III (NLS-II and NLS-III) are less well conserved and do not adhere as well to consensus nuclear localization signal sequences.

Two classes of nuclear localization signals are distinguished: monopartite and bipartite. The bipartite type of nuclear localization signals was first identified in Xenopus nucleoplasmin and has a **KR**PAATKKAGQA**KKK** sequence. It includes two basic residues preceding ten spacer residues that are in turn followed by a region with at least three basic residues (Dingwall et al., 1987). The first nuclear localization signal of p53, comprised of amino acids 305 thru 321, agrees with this consensus and has the following sequence: **KR**ALPNNTSSSPQP**KKK**. In contrast to this bipartite nuclear localization signal, the proposed monopartite nuclear localization signal consists of a K-K/R-X-K/R sequence, with the most often cited example being the SV40 large T-antigen NLS (PKKKRRV). Previous studies have proposed the existence of two other putative nuclear localization signals in the C-terminus of the p53 protein, although their sequences do not conform to the consensus sequence of either monopartite or bipartite nuclear localization signals. NLS II, is localized between amino acids 369 and 375 (LKSKKGQ), while NLS III resides between amino acids 379 and 384 (RHKKLM).

4. Purpose of the study

TP53 is one of the most frequently mutated genes of the genome. More than 50% of all cancers harbor a mutant p53 protein, and about 90% of cancers have alterations in the p53 pathway. These data underline the importance of the protein in protecting the genome. Mutations affecting the DNA-binding domain of the protein are the most common cause of a non-functional p53. In other cases, wild type p53 can be rendered non-functional due to cytoplasmic sequestration. Studying intracellular trafficking of p53 might give us a better understanding of p53 cytoplasmic accumulation, and ultimately a greater understanding of its role in some cancers.

In this study, KB-3.1 cells, a subclone of KB cells, were selected with either cisplatin or oxaliplatin. Incremental drug increases resulted in the isolation of one cisplatin and three oxaliplatin resistant cell lines designated KB-CP20, KB-OX20, KB-OX60 and KB-OX80. The three oxaliplatin-selected sublines were of the same lineage. Increased levels of p53 protein were detected in all the resistant sublines. In the oxaliplatin selected cell lines the p53 protein had a higher molecular weight. DNA sequencing identified in the KB-CP20 cells an acquired missense mutation (V172F) in the DNA binding domain and in the oxaliplatin selected cells a deletion of a single nucleotide in the sequence encoding for amino acid 382 in the putative nuclear localization signal III region. The deletion altered the putative NLS III and caused a frame-shift that resulted in a p53 protein that is 27 amino acids longer, and has been designated $p53^{420}$. Because previous studies have suggested that both NLS II and NLS III have supportive roles in the nuclear transport of p53, we explored the sub-cellular distribution of p53. Initial results suggested that in the oxaliplatin-selected sublines p53 was confined principally to the cytoplasm. To find an explanation for the relative cytoplasmic sequestration, we began by examining the steps that lead to the accumulation of p53 in the nucleus, such as p53 tetramerization, dynein binding, and trafficking through the nuclear membrane.

Intracellular trafficking plays an important role not only in the case of p53, but also in the activity of other important proteins such as BRCA, the androgen receptor,

and APC, proteins that are synthesized in the cytoplasm and function in the nucleus. A model of intracellular trafficking developed by the investigation of p53 could be used for studying these other molecules. The clinical significance of this is that microtubule targeting agents are effective in the clinic since they act mainly on interphase microtubules and partly because they target cells in mitosis. In my thesis I am going to show evidence that might lead to paradigm shift from a belief that microtubule-targeting agents prevent mitosis in patient tumors, to a novel concept that microtubule-targeting agents interfere with the function of interphase microtubules, such as intracellular trafficking.

5. Materials and methods

5.1. Cell lines and cell culture

Cisplatin-resistant and oxaliplatin-resistant cell lines were derived from the human cervical carcinoma cell line KB-3-1, a single clone of KB cells (a HeLa subclone). The platinum resistant lines were isolated by stepwise increases in the extracellular concentrations of either cisplatin or oxaliplatin. All cells were maintained in RPMI medium with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Briefly, cells were exposed initially to 0.25μM cisplatin or oxaliplatin, and a single clone was isolated from the few surviving cells. This clone was expanded and carried as a population during the subsequent selection process. The concentration of cisplatin or oxaliplatin was increased at intervals of several weeks to several months. The resistant cell lines used in these experiments were propagated in medium containing 20 μM cisplatin (KB-CP20) or 20, 60 or 80 μM oxaliplatin (KB-OX20, KB-OX60, KB-OX80). The three oxaliplatin-selected sublines were of the same lineage. Human prostate cancer cell line PC3 and human lung cancer cell line A549 were maintained in RPMI media supplemented with 10% FBS, 2 mM glutamine, 100 U / mL penicillin and 100 μg/mL streptomycin. Cells were kept at 37°C in 95% air and 5% CO_2.

5.2. Cytotoxicity Assay

Four-day cytotoxicity assays were performed using the sulforhodamine B based on the method of Skehan et al (Skehan et al., 1990). Briefly, cells were plated in 96-well, flat-bottom plates at a density of 1,500-2,000 cells/well and allowed to attach overnight at 37 °C in 5% CO_2. Chemotherapeutic agents at various concentrations were added to the cells and the plates were allowed to incubate for 96 hours at 37°C in 5% CO_2. Cells were subsequently fixed in 40% trichloroacetic acid. Plates were then washed, dried, and stained with sulforhodamine B solution (0.4% sulforhodamine B w/v in 1% acetic acid). After washing plates in 1% acetic acid and drying, the dye was solubilized in 10 mM Trisma base. The absorbances were read on a Bio-Rad plate reader at 540 nm. Each concentration was tested in triplicate. Relative resistance values were obtained by dividing the IC_{50} value of the parental KB-3-1 cell line for each drug by the IC_{50} value for the resistant cell lines.

5.3. Western Blot and Antibodies

Cell extracts were prepared in cell lysis in buffer containing 50 mM Tris (pH 7.5), 1% V/V NP-40 , 2 mM EDTA, 5M NaCl and Complete Mini, EDTA-free protease inhibitor cocktail tablets (Roche). Equal amount of protein pellets were re-suspended in 1x sample loading buffer and heated 99˚C for 5 minutes before being resolved by 10% SDS-polyacrylamide gels and immunoblotted on nitrocellulose membrane (Bio-Rad). The following antibodies were used: anti-p53 (Ab6, monoclonal Do1, Oncogene Science), tubulin (polyconal rabbit, Abcam), lamin B (polyclonal rabbit, Calbiochem) anti-dynein intermediate chain (IC74 monoclonal, Covance), anti-importin ß (31H4 monoclonal, Sigma), and anti-importin ß (ab2811, 3E9 monoclonal, Abcam), anti-PARP (polyclonal rabbit, Upstate, 06-557), HRP conjugated anti-mouse (NA931, Amersham), HRP conjugated anti-mouse kappa (1050-05, Southern Biotech), anti-mouse kappa (1050-01, Southern Biotech) labeled with SAIVI Alexa Fluor 680 with 0.1 mg labeling kit (Molecular Probes), anti-mouse IRDye 680 (926-32220, Li-cor), anti-rabbit IRDye 800CW (926-32211, Li-cor).

5.4. Subcellular Fractionation

Cells incubated overnight in 6-well plates at 80-90% confluence were harvested and fractionated using NE-PER nuclear and cytoplasmic extraction reagent (Pierce) according to the manufacturer's instruction. Samples were loaded in equal proportion on 10% SDS-PAGE and Western Blots were performed for p53 (monoclonal mouse anti-p53, 1:1000, Calbiochem), tubulin (polyconal rabbit anti-tubulin, 1:1000, Abcam), lamin B (polyclonal rabbit anti-lamin B, 1:500, Calbiochem) and PARP (polyclonal rabbit anti-PARP, 1:1000 Upstate). Densitometry values were obtained using the Odyssey Li-Core program.

5.5. Immunohistochemistry

Exponentially growing cells were plated on 12-mm glass coverslips and incubated overnight at 37 °C in 5% CO_2. The following day cells were rinsed in PBS and fixed with 3.7% formaldehyde/PBS for 10 min at room temperature. Cells were then fixed in 90% methanol for 5 min and coverslips were rinsed 2 times with PBS and incubated in blocking solution (5% donkey serum/PBS) for 30 minutes at room

temperature. Coverslips were rinsed 3 times in PBS between each of the steps. Primary and secondary antibodies were added sequentially for 1 h each at room temperature. The antibodies used were a sheep polyclonal (Ab7) antibody for p53 (Calbiochem) primary staining and a Texas RedX anti-sheep antibody as secondary antibody. DNA was counterstained with 1 g ml^{-1} 4,6-diamidino-2-phenylindole (DAPI, Sigma) in PBS.

5.6. Cross-linking Assay

For cross-linking experiments, 50-200 ug cell lysates were incubated with 10 mM diamide for 10 minutes at RT. Samples were heated at 99˚ for 5 minutes without a reducing agent and then resolved on a precast 4-15% gradient gel (Bio-Rad).

5.7. Immunoprecipitation

Cell lines or transiently transfected cells were lysed in IP buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, and Complete Mini, EDTA-free protease inhibitor cocktail tablets (Roche)), homogenized with a 25-gauge needle, and centrifuged at 14,000 rpm for 2 minutes at 4˚. Lysates (1 mg) were pre-cleared for 1 hr at 4˚ then incubated with IC74 anti-dynein or anti-importin ß (Abcam) overnight at 4˚. Protein and antibody were then incubated with ImmunoPure Plus Immobilized Protein A (Pierce) for 2 hours at 4˚. Samples were washed 5x with 500 ml of IP buffer. The Protein A pellet was re-suspended in 35 µl of 1x sample loading buffer and heated to 99˚ for 5 minutes before being resolved by 10% SDS-polyacrylamide gels and immunoblotted with antibodies for dynein, p53, or importin ß (Sigma). Results were determined by chemiluminescent detection (SuperSignal West Pico Chemiluminescent Substrate, Pierce) or by detection of IRDye infrared conjugated secondary antibodies using the LI-COR Odyssey.

5.8. Mutagenesis

Wild type and mutant p53 were amplified by polymerase chain reaction using a pcDNA3.1+ vector (Invitrogen) containing wt p53 as a template. For $p53^{K381T}$, $p53^{K382T}$ and $p53^{NLSIII}$ two end primers were designed. The 5’ primer contained a BamHI site, a Kozak sequence, and the ATG start codon of p53. The 3’ primer contained the point mutation(s), a stop codon, and an EcoRI site. For $p53^{FS382\text{-}393}$ the 3’ primer contained

the 382-393 amino acid sequence found in KB-OX cells, a stop codon, and an EcoRI site. One PCR was needed to generate the $p53^{K381T}$, $p53^{K382T}$, $p53^{NLSIII}$ and $p53^{FS382-393}$ mutants. For $p53^{NLSII}$ two end primers and two internal complementary primers were designed to introduce the mutations. Two primary PCRs were performed using the 5' end and the 3' internal primers or the 5' internal and the 3' end primers as sets. The products from the primary PCRs were annealed and used as template for the secondary PCR using the end primers to amplify the full-length product with the integrated mutations. For $p53^{NLSII/III}$, the $p53^{NLSII}$ was used as a template for the above mentioned 5' primer and the 3' primer containing the $p53^{NLSIII}$ mutations. For $p53^{420}$ four primers were designed: a 5' primer containing a BamHI site, a Kozak sequence, and the ATG start codon of the p53 gene, two internal primers complimentary to the 3' end of the $p53^{FS382-393}$ construct, and a 3' primer containing the mutation found in KB-OX cell lines, a stop codon, and an EcoRI site. Two primary PCR were performed using the $p53^{FS382-393}$ construct as a template or the RT product of the KB-OX80 cell line. The products from the primary PCRs were annealed and used as templates for the secondary PCR using the end primers to amplify the full-length product. All final PCR products were digested with BamHI and EcoRI before ligation into pcDNA3.1+ vector. DNA sequencing from both directions was used to confirm the sequence of the constructs.

5.9. Transfection

PC3 cells were plated in 6-well, flat-bottom plates at a density of 2×10^5 cells/well and allowed to attach overnight at 37 °C in 5% CO_2. The following day, the PC3 cells in RPMI were transiently transfected with p53 constructs using Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction. Following the 6 hour-long transfection period, sub-cellular fractionation was performed immediately or following drug treatment.

5.10. Luciferase reporter assays

70,000 PC3 cells/well in 12-well plates were transiently transfected using TransFast Transfection Reagent (Promega) for 48h. The transfection media (OPTI-MED, Gibco) was replaced with RPMI 5h after transfection. Vectors used included the backbone or the p21-Luc vector (kindly provided by Dr. Bert Vogelstein, Johns

Hopkins University), the pRL-TK internal control vector, and either the wt p53 vector or the $p53^{420}$ vector. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega).

6. Results and discussion

Part I. Characterization of a mutant p53 protein sequestered in the cytoplasm

6.1. Cell line characterization

Drug selection of the KB-3.1 human cervical cancer cell lines was performed in the laboratory of Dr. Tito Fojo (National Cancer Institute, NIH, Bethesda, MD, USA). The purpose of the selection was to achieve cell lines with high resistance to cisplatin or oxaliplatin for studying platinum drug resistance. The KB-3.1 cell line was chosen for drug selection based on the fact that chemotherapy regimens for cervical cancer often include either cisplatin or carboplatin. Moreover, KB-3.1, a subclone of the KB cell line has been used frequently in drug resistance studies. On the basis of cell characterizations, the KB cells appear to be a HeLa variant. Single clone isolates from KB-3.1 were obtained by exposure to 0.25 μM cisplatin or oxaliplatin. The cisplatin- and oxaliplatin-resistant KB-3.1 clones were expanded and exposed to gradually increasing concentrations of cisplatin or oxaliplatin, over a period of several months, before reaching the concentration of 20 μM cisplatin (KB-CP20) or 20, 60, 80 μM oxaliplatin (KB-OX20, KB-OX60, KB-OX80). The three oxaliplatin resistant cell lines are from the same lineage (Figure 11).

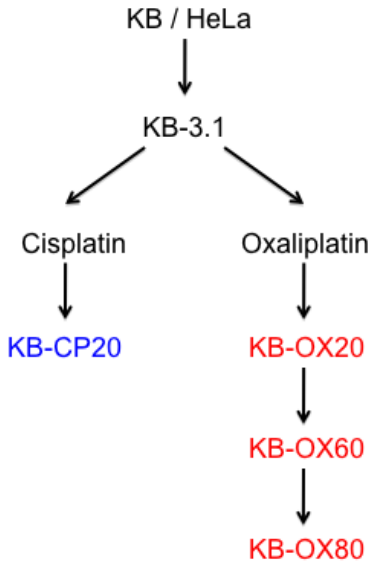


Figure 11. Selection of platinum resistant cell lines

In order to compare the sensitivity of the parental KB-3.1 cells to the KB-CP20, KB-OX20, KB-OX60 and KB-OX80 cell lines, cell survival was assessed following a four-day cisplatin, oxaliplatin or adriamycin treatment. Among the five cell lines, the KB-3.1 cell line is most sensitive to drug treatment. Upon cisplatin administration, the KB-CP20 cells were found to be the most resistant, followed by the oxaliplatin selected cell lines and the parental cell line. The KB-OX80 cell line was most resistant to oxaliplatin treatment, followed by the KB-OX60 cells. KB-OX20 and KB-CP20 cell lines possess a similar degree of resistance to oxaliplatin. Adriamycin, used as a control drug treatment, is comparably active in the parental and the resistant cells. The results of the drug sensitivity experiments are summarized in Table 3. The relative resistance (RR) was calculated as the IC_{50} of the drug in the resistant cell line, divided by the IC_{50} of the drug in the parental line. The result shows that the KB-CP20 cell line is more than 100-times more resistant to cisplatin, and the KB-OX60 cell line is almost 200-times more resistant to oxaliplatin compared to the parental cells.

Tabulation of absolute and relative resistance (μM)						
	Cisplatin IC_{50}	RR	**Oxaliplatin IC_{50}**	RR	**Adriamycin IC_{50}**	RR
KB-3.1	6.9×10^{7} M ± 0.1	1	9.0×10^{7} M ± 0.01	1	3.2×10^{8} M ± 0.03	1
KB-CP20	8.3×10^{5} M ± 14.3	120	4.4×10^{5} M ± 5.3	49	5.2×10^{7} M ± 0.04	16
KB-OX20	1.1×10^{5} M ± 1.0	16	5.7×10^{5} M ± 4.6	64	4.4×10^{8} M ± 0.01	1
KB-OX60	3.3×10^{5} M ± 4.9	48	1.4×10^{4} M ± 10.0	155	3.1×10^{8} M ± 0.03	1
KB-OX80	3.3×10^{5} M ± 1.0	48	2.5×10^{4} M ± 20.8	274	1.6×10^{7} M ± 0.06	5

Table 3. Absolute and relative resistance to cisplatin, oxaliplatin and adriamycin of the KB-3.1 and its resistant cell lines

6.2. Mutant p53 in the resistant KB cell lines

Platinum-based compounds cause DNA damage after entering the nucleus and binding to the DNA. Upon DNA damage signaling pathways eventually activate the p53

protein and this in turn initiates cell cycle arrest or apoptosis. Cell death caused by DNA-platination requires an intact p53 network. It is likely that cells resistant to platinum containing drugs develop a mechanism that renders p53 non-functional. A non-functional p53 can impair apoptosis leading to enhanced cell survival.

Cell lysates of KB-3.1 cells and its drug resistant sublines were probed for p53 protein. As shown in Figure 12, the level of p53 protein expression is barely detectable in the KB parental cell line. This suggests that KB-3.1 cells harbor a wild type p53. On the other had, increased levels of p53 protein were demonstrated in all drug resistant sublines. In the KB oxaliplatin selected cell lines not only was the protein expression increased, but also the molecular weight of the p53 was larger (The lower arrow in Figure 12 represents the size of wild type p53, and the upper arrow represents the size of p53 that was found in all oxaliplatin resistant sublines). A549 cells with wild-type p53 were used as a control.

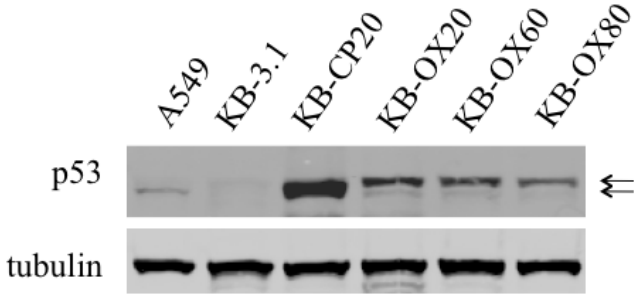


Figure 12. Protein expressions of p53 in the A549, KB-3.1 and its drug resistant sublines

DNA sequencing identified in the KB-CP20 cells an acquired missense mutation at amino acid 172, a part of the DNA binding domain, which changed the residue from valine to phenylalanine (V172F). Missense mutations in the DNA binding domain at amino acid 172 are among the most frequently occurring mutations in the p53 protein. DNA sequencing also revealed that the KB oxaliplatin selected cells harbor a deletion of a single nucleotide in the sequence encoding for amino acid 382 in the putative nuclear localization signal III (NLS III) region. The deletion altered the putative NLS III and caused a frame-shift that resulted in a p53 protein 27 amino acids longer than the

wild type designated $p53^{420}$. This confirms the larger size of p53 observed in the immunoblot analysis in the KB-OX cell lines. Since this deletion is present in all three oxaliplatin resistant sublines, one can assume it occurred early in the selection. The schematic structure of the wild type and mutant p53 found in the KB-3.1, KB-CP20 and KB-OX cell lines is shown in figure 13. The protein sequence of the wild type and mutant p53 found in the KB-3.1 and in the KB-OX cell lines are shown in Table 4.

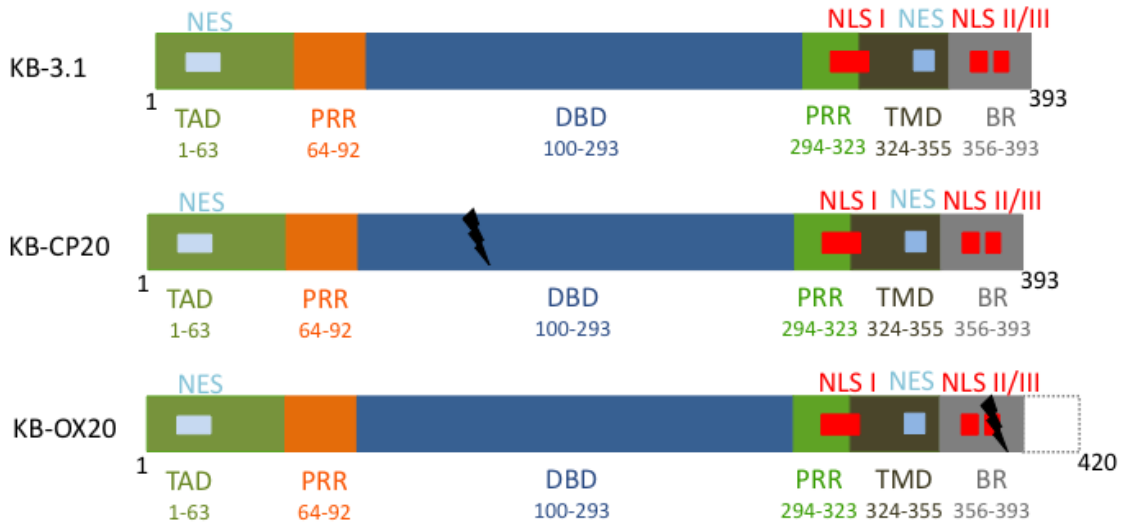


Figure 13. Schematic representation of the p53 proteins found in the KB-3.1 and its drug resistant cell lines

	382 393 420
Wild Type	H K K L M F K T E G P D S D -
KB-OX	H K **N S C S R Q K G L T Q T D I L H F L F P T D S L P P P S L P P L P F W V L G L** -

Table 4. Amino acid sequence of p53 proteins in the parental KB-3.1 and its oxaliplatin resistant cell lines

6.3. Nuclear localization of $p53^{420}$

Since the deletion detected in the KB-OX cell lines has never been reported before in the literature, we began to investigate the function of the mutant $p53^{420}$ protein. Previous studies have suggested that p53 has three putative nuclear localization signals, and among those NLS I is the most important while NLS II and NLS III have

been suggested to have supportive roles in the nuclear translocation of the protein. Our aim was to explore the sub-cellular distribution of the $p53^{420}$ possessing an altered NLS III signal and 27 ≤extra amino acids on the C-terminus of the protein. We extracted nuclear and cytoplasmic proteins from parental KB cells and its resistant sublines before and after the administration of the DNA-damaging agent, adriamycin. Figure 14 shows an immunoblot of the subcellular distribution of p53 in the A549, KB-3.1 and its drug resistant cell lines. The quality of the separation was assessed using tubulin and PARP as markers of the cytoplasmic and nuclear fractions, respectively. As a control, the A549 cell line with wild type p53 was used. The adriamycin treatment did not change significantly the p53 protein expression levels in the cell lines harboring mutant p53, although it did increase the expression of the wt p53 in A549 cells. Furthermore, while treatment with adriamycin increased the fraction of p53 in the nucleus in the A549 cell line, it had no effect on the sub-cellular distribution of mutant p53 in the cisplatin and in the oxaliplatin resistant cells. Approximately 60% of the p53 protein in KB-CP20 cells localized to the nucleus, compared with a predominant cytoplasmic distribution (85%) of the mutant p53 found in the oxaliplatin selected cell lines. Thus it appeared that the oxaliplatin-selected sublines harbored a p53 mutation that altered NLS III and led to its sequestration in the cytoplasm.

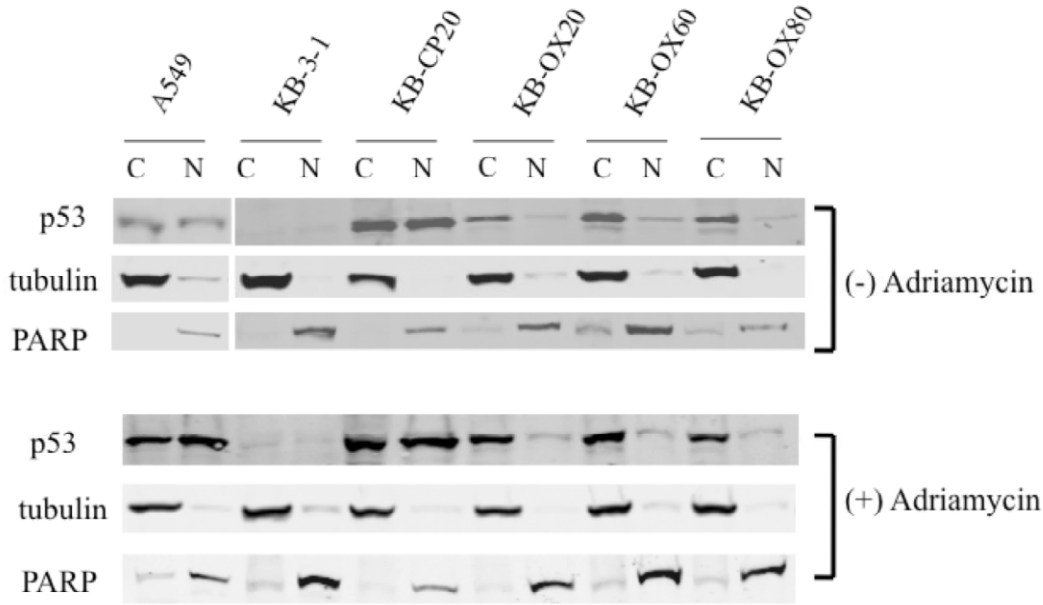


Figure 14. Subcellular distribution of p53 protein in A549, KB-3.1 and its drug resistant sublines

6.4. Microtubular trafficking of $p53^{420}$

We sought possible explanations for the relative cytoplasmic sequestration seen in the oxaliplatin-selected cells. We began by examining the steps in the cytoplasm that lead to accumulation of p53 in the nucleus. Earlier studies have demonstrated that p53 oligomerizes in order to bind dynein, the negative-end motor protein of the microtubules. To examine the ability of $p53^{420}$ to oligomerize, we performed experiments with the cross-linking reagent, diamide. As a positive control, we used A549 lung carcinoma cells that harbor a wild type p53 and had been treated with adriamycin so as to raise the endogenous levels of p53. As shown in figure 15, in the adriamycin treated A549 cells, as well as all the oxaliplatin-selected cell lines, cross-linked p53 oligomers were demonstrated as discrete bands on an immunoblot in the diamide treated samples.

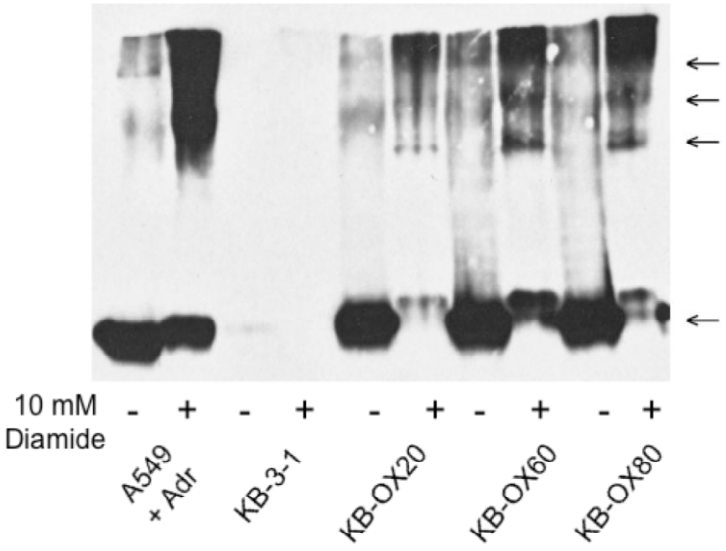


Figure 15. Immunoblot of the p53 oligomerization in the A549, KB-3.1 and its drug resistant cell lines

Oligomerization of p53 is necessary for dynein association and this association is independent of microtubules. Further evidence of the existence of such oligomers was obtained by demonstrating the co-immunoprecipitation of dynein and p53 as shown in figure 16. Both $p53^{420}$, found in the oxaliplatin-selected cell lines, and $p53^{V172F}$, found in cisplatin-selected KB-CP20 cells, could be immunoprecipitated with dynein. This finding was further confirmed by transfecting PC3 prostate cancer cells that lack p53,

with either wild type or mutant p53 protein prior to performing the co-immunoprecipitation. $p53^{420}$ is the original mutation found in the KB-OX cell lines, while $p53^{FS382\text{-}393}$ harbors the same deletion at amino acid 382, the same frame-shift, but the protein length is truncated at amino acid 393. As shown in the lower panel of figure 16 in all cases the transfected p53 protein co-precipitated with dynein. Thus we concluded that the preferential cytoplasmic sequestration of $p53^{420}$ is not a consequence of impaired dynein binding or impaired trafficking on microtubules.

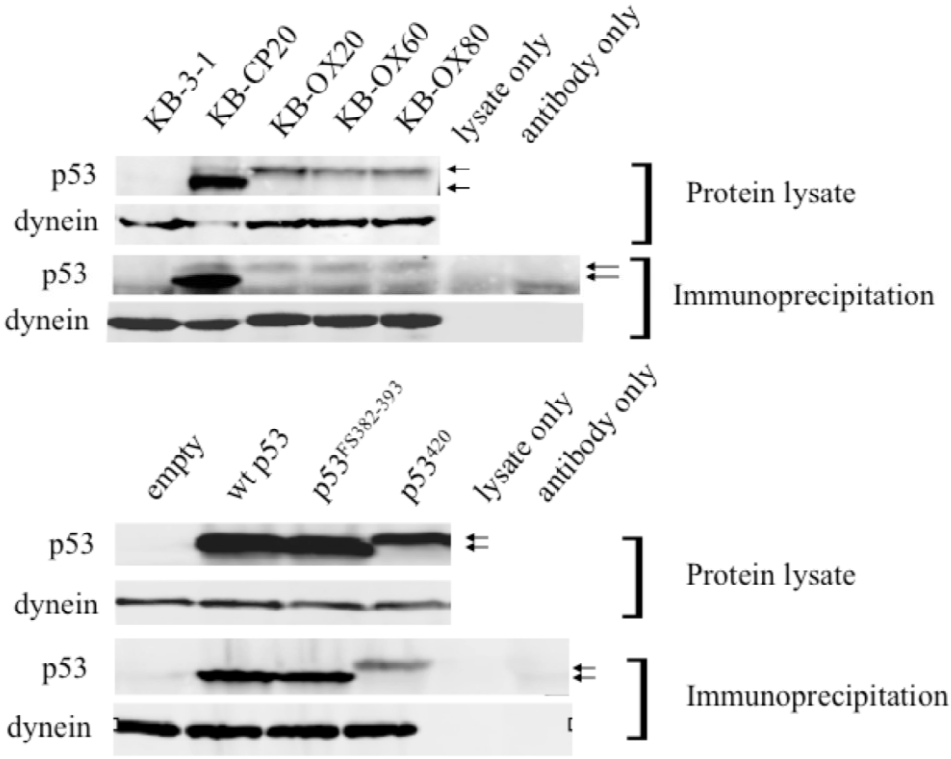


Figure 16. Co-immunoprecipitation of p53 and dynein

6.5. Augmented nuclear export is not the reason of $p53^{420}$ cytoplasmic accumulation

Wild-type p53 is rendered non-functional in some colorectal cancers, inflammatory breast cancers, melanoma malignum and undifferentiated neuroblastomas because of its cytoplasmic sequestration (Bosari *et al.*, 1995; Lilling *et al.*, 2002; Moll *et al.*, 1995; Weiss *et al.*, 1995). Impaired nuclear translocation and increased nuclear export can lead to cytoplasmic sequestration. To exclude the possibility that $p53^{420}$

accumulates in the cytoplasm secondary to increased nuclear export, we sought to investigate the effect of leptomycin B, a drug that blocks the exportin 1 function, on the sub-cellular distribution of p53 in the KB-OX cell lines. Leptomycin B disrupts covalent bonds between the cargo and exportin 1, the carrier protein responsible for p53 nuclear export. KB-CP20 and KB-OX60 cells were treated with 10 ng/ml leptomycin B followed by immunohistochemical staining for p53. As the upper part of Figure 17 shows, leptomycin B treatment increased the intensity of p53 nuclear staining in the KB-CP20 cell line ($p < 0.001$ comparing KB-CP20 ± Leptomycin B), but had no effect in the KB-OX60 cell line. The bottom part of figure 15 shows a bar graph of the nuclear intensity of p53 in the KB-CP20 and the KB-OX60 cell lines after measuring the intensity of 300 nuclei per samples. In both cell lines, the untreated samples are arbitrarily assigned a value of 100%. This data indicates the preferential cytoplasmic localization of $p53^{420}$ is not a result of augmented nucleo-cytoplasmic transport.

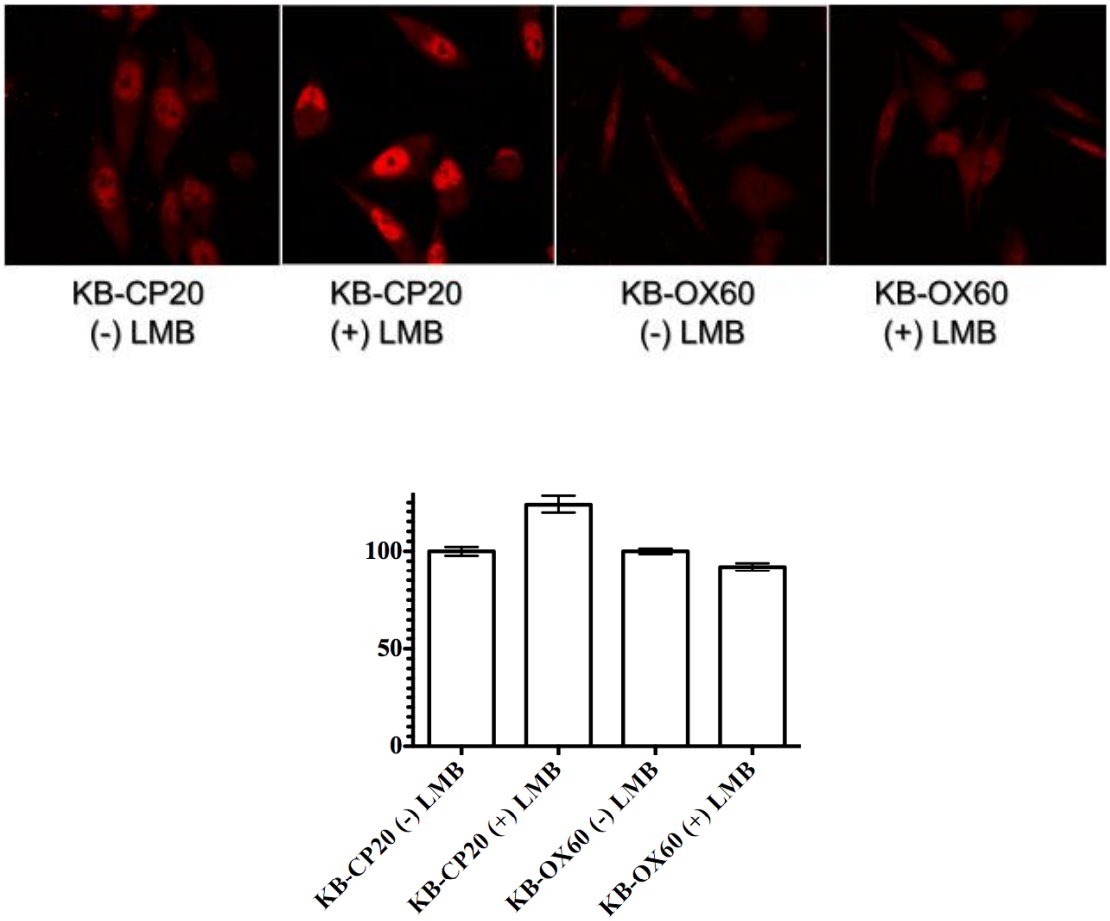


Figure 17. Nuclear intensity of p53 in the KB-CP20 and in the KB-OX60 cell lines

6.6. Neither NLSII nor NLSIII are required for p53 nuclear import

It is thought that p53 has three putative nuclear localization signals, designated as NLS I (residues 305-322), NLS II(residues 369-375) and NLS III (residues 379-384). NLS I has been reported as the main nuclear localization signal responsible for p53 nuclear translocation (Shaulsky *et al.*, 1990). It has been assumed that NLS II and NLS III have supportive roles in this process. Given the fact that $p53^{420}$, possessing a destroyed NLS III sequence, is retained in the cytoplasm, we decided to better assess the role of NLS II and NLS III in p53 nuclear translocation. To evaluate the importance of these signals in p53 nuclear import, nine p53 constructs were generated with either one or both NLS II and NLS III destroyed and of either normal length or 420 amino acids in length - the length of $p53^{420}$. The nine constructs included: (1) $p53^{K381T}$, (2) $p53^{K382T}$, (3) ΔNLSIII (p53K381T/K382T), (4) $p53^{FS382\rightarrow393}$ (FS = frame shift), (5) $p53^{420}$, (6) ΔNLSII (p53K372T/K373T), (7) ΔNLSII/III ($p53^{K372T/K373T,\ K381T/K382T}$), (8) p53 (4G), (9) wt p53, as well as a vector control. The sequences of the nine constructs are shown in table 5. The control constructs included wt p53 as well as p53 (4G), a construct in which amino acid residues F338G, R342G, L344G and E346G are replaced with glycines. In previous studies it has been demonstrated that p53 (4G) is unable to oligomerize and associate with dynein, and as a consequence it is unable to translocate to the nucleus.

	NLSII	...	NLSIII
Wild Type	K S K K G Q	...	H K K L M F K T E G P D S D –
KB-OX	K S K K G Q	...	H K **N S C S R Q K G L T Q T D I L H F L F P T D S L P P P S L P P L P F W V L G L**
$p53^{K381T}$	K S K K G Q	...	H **T** K L M F K T E G P D S D –
$p53^{K382T}$	K S K K G Q	...	H K **T** L M F K T E G P D S D –
ΔNLSIII	K S K K G Q	...	H **T T** L M F K T E G P D S D –
$p53^{FS382-393}$	K S K K G Q	...	H K **N S C S R Q K G L T Q T** –
$p53^{420}$	K S K K G Q	...	H K **N S C S R Q K G L T Q T D I L H F L F P T D S L P P P S L P P L P F W V L G L**
ΔNLSII	K S **T T** G Q	...	H K K L M F K T E G P D S D –
ΔNLSII/III	K S **T T** G Q	...	H **T T** L M F K T E G P D S D –
4G	Impaired tetramerization as a consequence of four glycine substitution: F338G, R342G, L344G, E346G		

Table 5. Sequences of the nine p53 constructs used in the transfection experiments

For transfection, the PC3 prostate cancer cell line was selected since it does not express endogenous p53 protein. Following transfection the distribution of p53 was determined by sub-cellular fractionation as shown in the upper part of figure 18. The nuclear fraction of p53 in each sample (%N) is shown as percentage and calculated as

%N = [N/(C+N)] x 100. The quality of the separation was assessed using tubulin and lamin B as markers of the cytoplasmic and nuclear fractions, respectively. Quantitations were performed using the results from three different transfections and 2 immunoblots from each, and the result is shown in the lower part of figure 16. Impaired nuclear accumulation was observed only with the p53 (4G) and the $p53^{420}$ constructs. This result indicates that neither NLSII nor NLSIII alone or in combination are essential for the nuclear translocation of p53. Thus the cytoplasmic sequestration of $p53^{420}$ cannot be ascribed to destruction of NLSII or NLSIII.

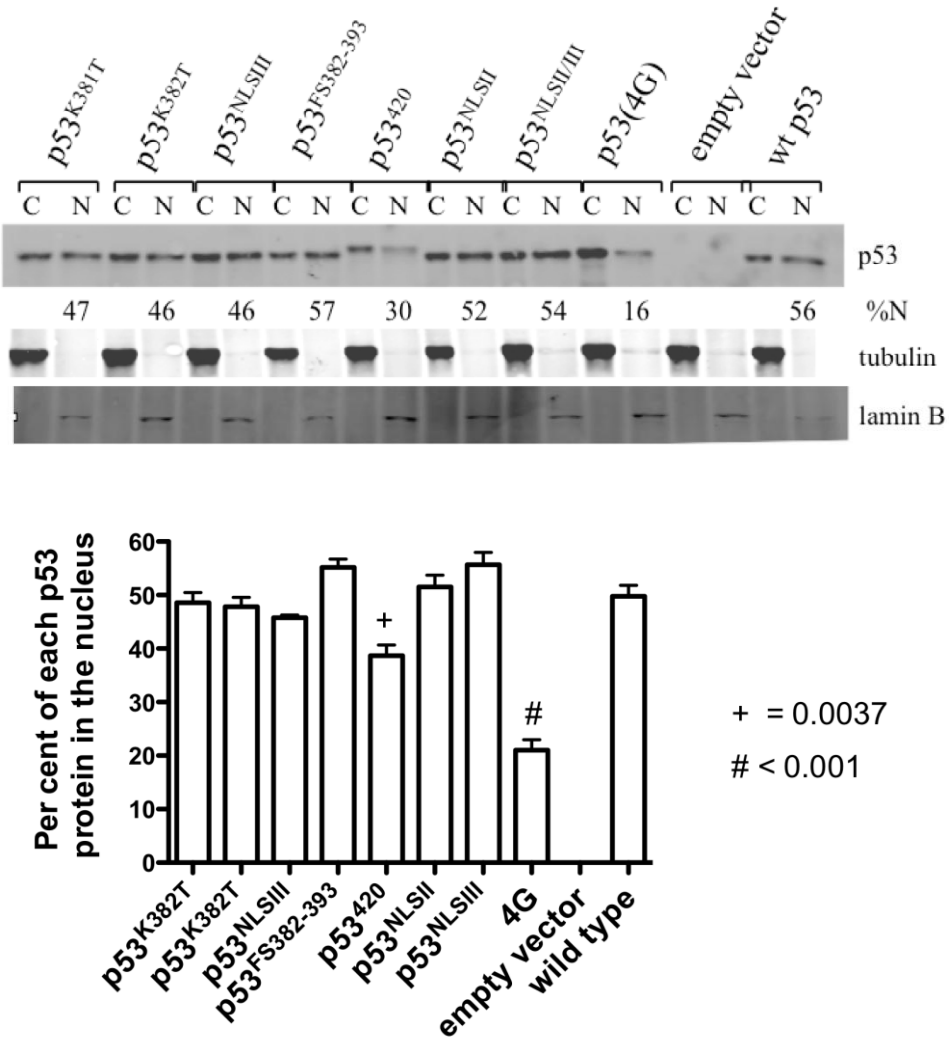


Figure 18. Cellular distribution of mutant p53 proteins in PC3 cells following transfection

6.7. Rapid degradation leads to reduced stability of $p53^{420}$

During the course of immunoblot evaluations, we had observed that cells transfected with $p53^{420}$ had a lower intensity of protein bands compared to the wild type or other mutant proteins suggesting that $p53^{420}$ is expressed at a lower level. We considered the possibility that the more rapid degradation of $p53^{420}$ might be responsible for the reduced stability and subsequently for the apparently lower protein levels. To assess the stability of the $p53^{420}$, PC3 cells transiently transfected with either $p53^{420}$ or wild type p53 vectors were treated with the protein synthesis inhibitor, cycloheximide for variable times prior to harvesting protein. Cycloheximide, a widely used inhibitor of eukaryotic protein biosynthesis, rapidly and reversibly blocks protein translation. The results are shown in figures 19 and 20. The level of $p53^{420}$ remained stable without drug administration; however, it declined rapidly after cycloheximide treatment. As shown in figure 19, the $p53^{420}$ protein level has decreased by 60% at the end of the two-hour long drug treatment. By comparison, the levels of wild type p53 appeared more stable in this system. Figure 20 shows that the expression levels of wild type p53 remained stable in this short incubation period, even though cycloheximide was added and increased if cycloheximide was not administered. This increase in the expression level is not unexpected following a transfection since levels usually increase over time.

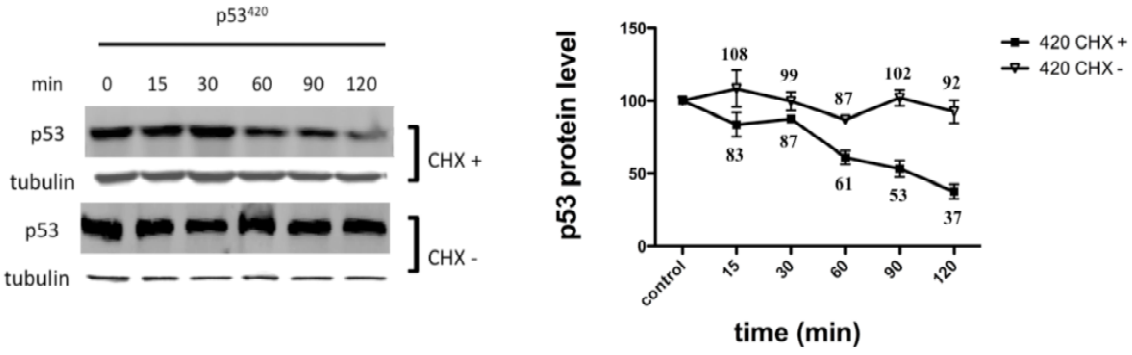


Figure 19. Proteasomal degradation of mutant p53 ($p53^{420}$) transfected into PC3 cells

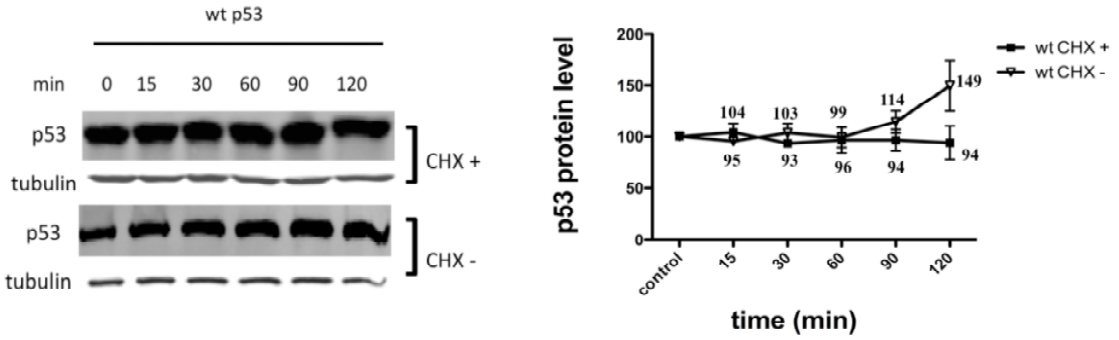


Figure 20. Proteasomal degradation of wild type p53 transfected into PC3 cells

To confirm this finding, we examined the stability of p53 in KB-OX60 and KB-CP20 cells. Cell lysates from these cell lines were extracted after variable times of cycloheximide treatment up to 5 hours. In the presence of cycloheximide, the level of $p53^{420}$ in the KB-OX60 cells decreased more rapidly than that of $p53^{V172F}$ in the KB-CP20 cells. Figure 21 shows that during the five-hour treatment the protein level of $p53^{V172F}$ decreased by about 50%, while the protein level of $p53^{420}$ decreased by approximately 80% compared to control levels. Notably, a two-hour cycloheximide treatment of KB-OX60 cells decreased the p53 protein level to 44% of the original level, a value that correlates well with the decrease detected in the $p53^{420}$ transfected PC3 cells (37%). One explanation for these results is the possibility that the additional 27 amino acids at the C-terminus of $p53^{420}$ destabilize the protein to some extent; however, destabilization is not to an extent sufficient to preclude accumulation of the mutant protein in these cells to levels much higher than those found in parental KB-3.1 cells that harbor a wt p53. Alternatively, it is possible that simply because degradation of p53 occurs primarily in the cytoplasm (Freedman and Levine, 1998; Roth et al., 1998) and $p53^{420}$ is primarily confined to the cytoplasm, the total levels fall more rapidly.

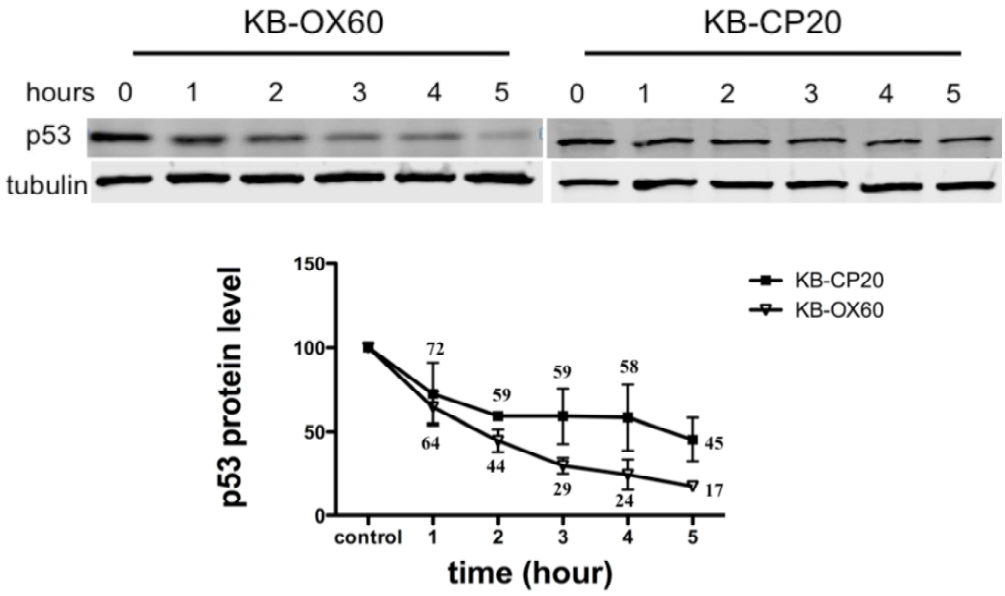


Figure 21. Proteasomal degradation of p53 in the KB-CP20 and KB-OX60 cell lines

6.8. Association between $p53^{420}$ and importin β

Having excluded impaired oligomerization, altered dynein binding and intracellular trafficking, augmented nuclear export, and a role for either NLS II or NLS III in nuclear translocation, we next assessed whether altered association to the importin-α/β complex with reduced nuclear import might explain the cytoplasmic accumulation $p53^{420}$. In the literature we found only indirect evidence for the role of importin in the transport of most proteins and no direct evidence for a role in the transport of p53 (Kim *et al.*, 2000; Li *et al.*, 2007; Liang & Clarke, 1999). We conducted immunoprecipitation experiments to assess the extent of importin association with p53 and its putative role in the nuclear transport of p53 by more accurately quantitating the extent of the association of p53 with importin-α/β. KB-CP20 (harboring $p53^{V172F}$) and A549 (possessing wild type p53) cell lines were chosen as controls. As shown in the upper panel of figure 22, the association of $p53^{420}$ with importin was impaired in the KB-OX60. By comparison, the association with importin was not impaired in either KB-CP20 cells ($p53^{V172F}$) or A549 cells treated with adriamycin (wt p53). In the immunoprecipitation experiment the ratio between importin-β and the p53 brought down in the immunoprecipitation was comparable for the A549 and KB CP20 cell lines. In contrast, despite higher expression levels of importin-β in the KB-OX60

cells less $p53^{420}$ was immunoprecipitated from the cellular lysates. We thus concluded $p53^{420}$ is sequestered in the cytoplasm because it is excluded from entering the nucleus as a consequence of poor association with importin.

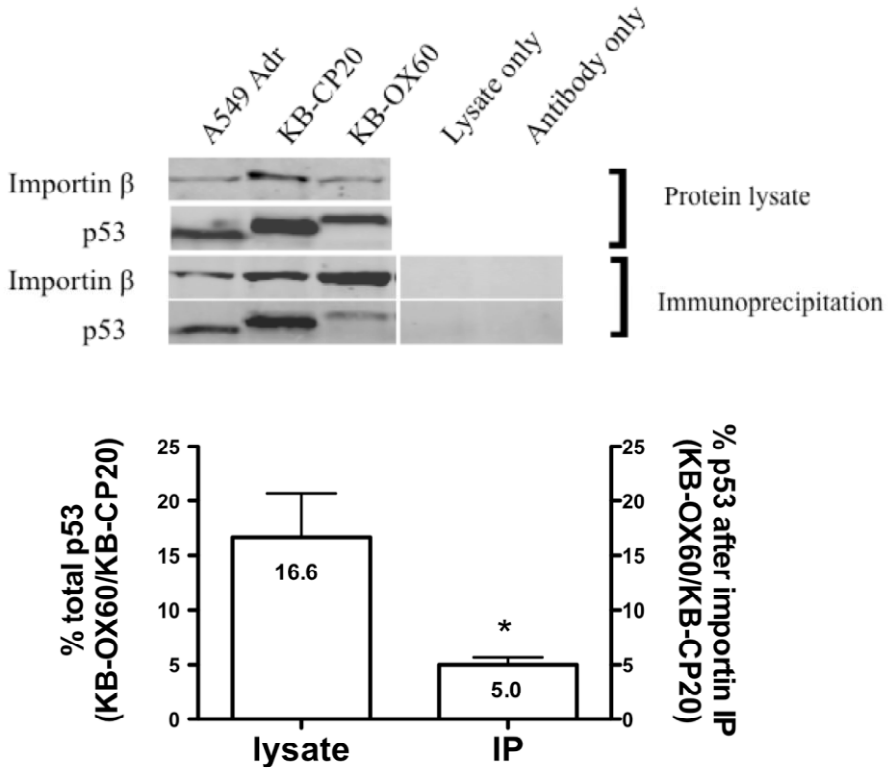


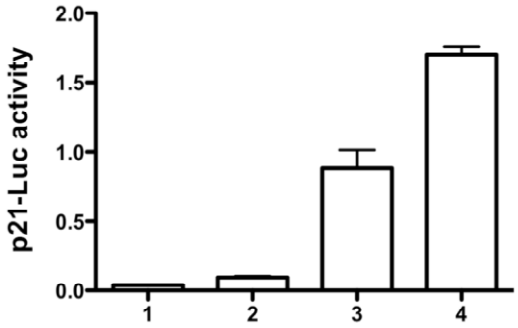
Figure 22. Co-immunoprecipitation of p53 and importin-β

6.9. The trans-activation ability of $p53^{420}$ remained intact

The role of p53 following DNA damage is to bind to DNA and transactivate genes involved in cell cycle arrest and apoptosis. A mutation altering the DNA binding domain of p53, such as $p53^{V172F}$ found in cisplatin selected KB cells, interferes with the ability of the protein to trans-activate target genes and protect the genome. This type of mutant protein can "safely" cross the nuclear membrane since it cannot effectively trans-activate target genes and endanger the survival of cancer cells. To reveal the advantage of cytoplasmic sequestration of $p53^{420}$, we conducted experiment to assess the ability of this mutant protein to transactivate promoters normally activated by p53. To this end, PC3 cells were co-transfected with a p21-vector and a vector containing a mutant or wild type p53 (wild type as a control). The p21-vector includes the sequence

specific-DNA binding site for p53 and has luciferase activity. A p53 protein that can bind to DNA, can induce luciferase activity in the p21-vector.

As shown in figure 23, $p53^{420}$ stimulated luciferase activity indicating this mutant vector can trans-activate a p53-inducible promoter. We thus conclude that $p53^{420}$ retains the ability to trans-activate target genes, in this case p21, and hence its sequestration in the cytoplasm is essential to inhibit its activity. Thus in the case of $p53^{420}$ impaired nuclear import precludes a protein that can potentially trans-activate genes from reaching its targets achieving a similar outcome to that most commonly occurring in cells harboring mutant p53 proteins – an impaired ability to trans-activate target genes.



	1	2	3	4
p21-basic-Luc	+	+		
p21-Luc			+	+
pRL-TK	+	+	+	+
Wt-p53	+		+	
$p53^{420}$		+		+

Figure 23. Luciferase activity of mutant and wild type p53 co-transfected into PC3 cells with a p21 luciferase reporter

In summary, the study on the KB-3.1 cell line and its drug resistant cells showed that the KB-CP20 cell line harbors a frequently occurring mutation in the TP53 gene. This mutation, a missense mutation that changed amino acid 172 from valine to phenylalanine (V172F) alters the DNA-binding domain of the protein leading to a non-functional p53. The KB-OX cell lines harbor a unique mutant p53 protein. A deletion of a single nucleotide in the sequence encoding for amino acid 382 alters the nuclear localization signal III and results in a frame-shift mutation leading to a protein designated as $p53^{420}$ that is 27 amino acids longer than wt p53. This mutant $p53^{420}$ is sequestered primarily in the cytoplasm, although it is able to tetramerize and bind to dynein, the negative motor protein of the microtubules. We demonstrated that the

reduced nuclear accumulation of $p53^{420}$ is not the consequence of an augmented nuclear export. Site directed mutagenesis showed that the p53 nuclear localization signals II and III are not essential for nuclear translocation of the protein. We also demonstrated that the mutation in $p53^{420}$ confers a relative instability to the protein, although the levels of the protein in the oxaliplatin resistant cells are high. The data above showed that the poor interaction of $p53^{420}$ with importin is the explanation for the retention of $p53^{420}$ in the cytoplasm. $p53^{420}$ cytoplasmic sequestration is advantageous for the cancer cells, because this mutant p53 retains the ability to transactivate p53-inducible genes such as p21.

Part II. Microtubule targeting agents and mitotic kinase inhibitors: Understanding how they work and the clinical implications

What is the clinical implication of (p53) intracellular trafficking in cancer treatment? In the following paragraphs I am going to show evidence, supported by the observation that microtubule targeting agents cause mitotic arrest in cell culture, that the clinical role of interphase microtubules (such as intracellular trafficking) has been underestimated and the presence of cell division in human cancer has been overstated. This misinterpretation has led to the development of a whole new class of kinase inhibitors in the era of targeted therapy. However, the lack of effectiveness of mitotic kinase inhibitors in clinical trials have now ratified the paradigm that drugs targeting the microtubules have been successful clinically not only by interfering with mitosis but more importantly by disrupting microtubule trafficking.

6.10. Microtubule targeting agents

Microtubule targeting agents have been divided into two classes: 1) polymerizing agents and 2) depolymerizing agents (Table 5). Both types of drugs suppress the dynamics of the microtubules. Polymerizing agents (e.g. taxol) stabilize the microtubules, while depolymerizing agents (e.g. vincristine) prevent microtubules polymerization. They are widely used as a first line therapy in the treatment of a variety of human cancers such as breast cancer, colorectal cancers, ovarian cancer, prostate cancer and lymphomas (Table 5).

I. Microtubule polymerizing agents		
Taxanes	Taxol	Breast cancer, colorectal cancer, ovarian cancer
	Docetaxel	Breast cancer, non-small cell lung cancer, prostate cancer, gastric adenocarcinoma, head and neck cancer
Epothilones	Ixabepilone	Breast cancer, non-small cell lung cancer, prostate cancer
II. Microtubule depolymerizing agents		
Vinca alkaloids	Vinblastine	Hodgkin's disease, lymphoma, testicular cancer
	Vincristine	Neuroblastoma, Hodgkin's disease, non-Hodgkin's disease, AML, Wilms' tumor
	Vinorelbine	Non-small cell lung cancer
Halichondrin B	Eribulin	Breast cancer

Table 5. Classification of microtubule targeting agents

To date, the proposed mechanism of action of microtubule targeting agents that has been most widely accepted is the inhibition of cell proliferation as a consequence of microtubule disruption. The evidence that these drugs inhibit mitosis comes exclusively from *in vitro* and xenograft models (Horton et al., 1988; Milas et al., 1995). Primary tumor cells undergo selection in order to establish consistent cell lines with extended replicative capacity that can be used for *in vitro* culture and *in vivo* models. During immortalization the doubling time of primary tumor cells significantly decreases; *in vitro*, doubling times are usually less than 24 hours. For example, in the sixty cell lines of the NCI anti-cancer drug screen, the median in vitro doubling time is 33.1 hours and the range is 17.4 (HCT-116) to 79.5 (HOP-92) hours (mean: 35.67 hours, median: 33.1 hours) [NCI Developmental Therapeutics program webpage http://dtp.nci.nih.gov/docs/misc/common_files/cell_list.html]. These cell lines have slightly longer doubling times as xenograft models as they ranges from 1.2 days to 12.2 days [mean: 4.7 days (113 hours), median: 4.3 days (103 hours)] (Teicher, 1997) (Table 6).

Cell Line Name	Panel Name	Doubling Time In vitro (hours)	Doubling time In vivo xenograft (days, mean)
CCRF-CEM	Leukemia	26.7	4.6
HL-60(TB)	Leukemia	28.6	3.3
K-562	Leukemia	19.6	NA
MOLT-4	Leukemia	27.9	1.2
RPMI-8226	Leukemia	33.5	NA
SR	Leukemia	28.7	5.1
A549/ATCC	Non-Small Cell Lung	22.9	8.4
EKVX	Non-Small Cell Lung	43.6	5.5
HOP-62	Non-Small Cell Lung	39.0	3.6
HOP-92	Non-Small Cell Lung	79.5	6.0
NCI-H226	Non-Small Cell Lung	61.0	NA
NCI-H23	Non-Small Cell Lung	33.4	3.7
NCI-H322M	Non-Small Cell Lung	35.3	4.0
NCI-H460	Non-Small Cell Lung	17.8	2.1
NCI-H522	Non-Small Cell Lung	38.2	2.3
COLO 205	Colon	23.8	4.3
HCC-2998	Colon	31.5	3.5
HCT-116	Colon	17.4	2.6
HCT-15	Colon	20.6	3.4
HT29	Colon	19.5	5.1
KM12	Colon	23.7	2.4
SW-620	Colon	20.4	2.4
SF-268	CNS	33.1	NA
SF-295	CNS	29.5	1.4
SF-539	CNS	35.4	8.4
SNB-19	CNS	34.6	6.9
SNB-75	CNS	62.8	3.1
U251	CNS	23.8	4.3
LOX IMVI	Melanoma	20.5	1.5
MALME-3M	Melanoma	46.2	11.2
M14	Melanoma	26.3	6.7
MDA-MB-435	Melanoma/Breast	25.8	6.6
SK-MEL-2	Melanoma	45.5	5.7
SK-MEL-28	Melanoma	35.1	1.9
SK-MEL-5	Melanoma	25.2	7.3
UACC-257	Melanoma	38.5	5.4
UACC-62	Melanoma	31.3	2.8
IGR-OV1	Ovarian	31.0	6.4
OVCAR-3	Ovarian	34.7	5.5
OVCAR-4	Ovarian	41.4	6.2
OVCAR-5	Ovarian	48.8	3.3
OVCAR-8	Ovarian	26.1	12.2
NCI/ADR-RES	Ovarian	34.0	-
SK-OV-3	Ovarian	48.7	3.4

786-0	Renal	22.4	6.7
A498	Renal	66.8	3.4
ACHN	Renal	27.5	NA
CAKI-1	Renal	39.0	2.1
RXF 393	Renal	62.9	3.4
SN12C	Renal	29.5	5.6
TK-10	Renal	51.3	NA
UO-31	Renal	41.7	NA
PC-3	Prostate	27.1	2.4
DU-145	Prostate	32.3	4.4
MCF7	Breast	25.4	4.5
MDA-MB-231/ATCC	Breast	41.9	4.4
MDA-MB-468	Breast	62	-
HS 578T	Breast	53.8	NA
MDA-N	Breast	22.5	7.9
BT-549	Breast	53.9	NA
T-47D	Breast	45.5	NA
Range Mean Median		17.4-79.5 35.67 33.1	1.2-12.2 4.66 4.3

Table 6. Tumor doubling time of the NCI 60 cell lines in vitro and in vivo

In a rapidly dividing cell line the consequence of adding a microtubule-targeting agent is mitotic arrest. For example, 100 nM taxol treatment of the A549 cell line results in mitotic arrest, detected on FACS analysis as G2/M arrest 24 hours following the administration of drug (Figure 24).

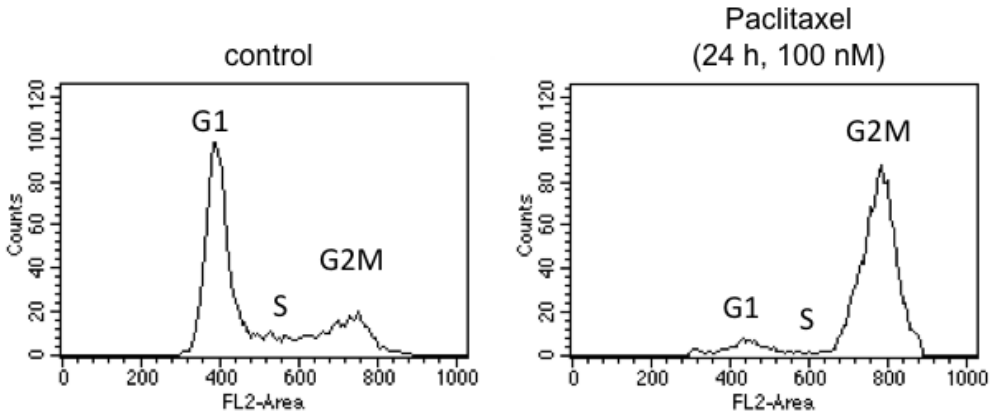


Figure 24. Rapid G2/M arrest following paclitaxel treatment

Similar results are observed in xenograft models. Mice bearing MDA-MB231 xenograft treated with 2-methoxyestradiol (2-ME) rapidly undergo mitotic arrest. As shown on the left panel of Figure 25, the majority of cells display abnormal mitotic spindles 24 hours after treatment (Mabjeesh *et al.*, 2003).

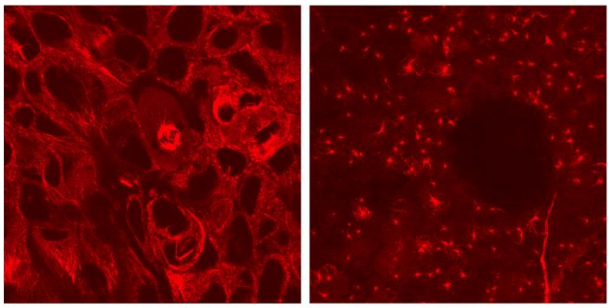


Figure 25. MDA-MB231 xenograft model 24 hours after treatment with a microtubule targeting agent. Adapted from Mabjeesh NJ et al. Cancer Cell 3:363-75 2003

Based on these preclinical data, the mechanism of cytotoxicity of microtubule-targeting agents has been attributed to the induction of mitotic arrest. The clinical success and the occurrence of neurotoxicity with these agents lead to the search for more mitosis-specific drugs, which in turn produced the mitotic kinase inhibitors. The expectation was that the mitotic kinase inhibitors would be as good as if not better than microtubule-targeting agents but without major side effects, most notably without neurotoxicity (Harrison et al., 2009; Jackson et al., 2007; Nakai et al., 2009). Since mitotic kinase inhibitors target cells in mitosis only, and neuron cells do not divide, the expectation was that neurotoxicity would not develop in patients treated with this class of drugs.

6.11. Mitotic kinase inhibitors

The past decade has brought a greater understanding of the complex choreography of mitosis, as proteins responsible for different functions have been identified and characterized (Schmit & Ahmad, 2007). In the synchronization of mitosis we gained a clearer view of the fact that many other proteins besides tubulin are crucial. Many of these newly described proteins are only functional during mitosis and are responsible for controlling different steps in the assembly and function of the complex machinery of the mitotic spindle (Figure 26) (Crosio et al., 2002). Based on the believe

that cancer cells grow more rapidly and might be more vulnerable to mitotic directed agents,, these proteins, including the aurora kinases and the polo-like kinases emerged as attractive targets for cancer treatment (Chopra *et al.*; Colombo & Moll, 2008; Taylor & Peters, 2008).

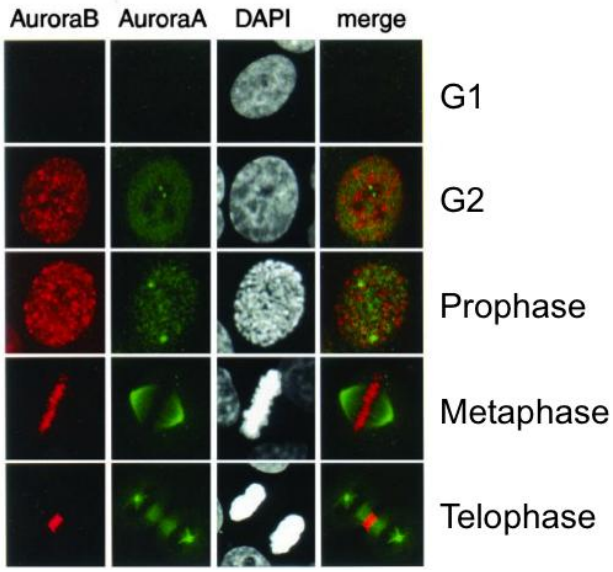


Figure 26. The expression of aurora A and aurora B is limited for mitosis
Adapted from Crosio et al., 2002

Aurora A localizes to the centrosome in the S-phase of the cell cycle (Dutertre et al., 2002). As the cell progresses through prophase, metaphase, and anaphase the level of Aurora A increases and the protein translocates to the mitotic poles and the adjacent spindle microtubules (Figure 27)(Crosio *et al.*, 2002). During telophase and cytokinesis, Aurora A is located in the midbody. Aurora B and Aurora C are chromosome passenger proteins (Sasai et al., 2004). While Aurora B localizes to microtubules near kinetochores (especially to K-fibers, the specialized microtubules), Aurora C moves from the centromeres to the midzone of mitotic cells as the cell cycle progresses.

Polo-like kinases (PLK) localize to the centrosome during interphase and prophase, move to the spindle poles following prophase, and relocate to the midbody for telophase and cytokinesis (Kishi *et al.*, 2009; van de Weerdt & Medema, 2006) (Figure 27).

Kinesin spindle protein, like the mitotic kinases, is mitosis-specific and hence has attracted attention as a target for mitosis-directed anti-cancer agents. Kinesin spindle protein is a homotetrameric kinesin motor protein whose anti-parallel microtubules-sliding function is required for separation of the two centrosomes in early mitosis. Since centrosome separation is essential for bi-polar spindle formation, inhibition of kinesin spindle protein results in mitotic arrest due to formation of a MT array with only one pole. A number of kinesin spindle protein inhibitors have been described and some have progressed to clinical trials (Huszar *et al.*, 2009; Sarli & Giannis, 2008; Zhang & Xu, 2008).

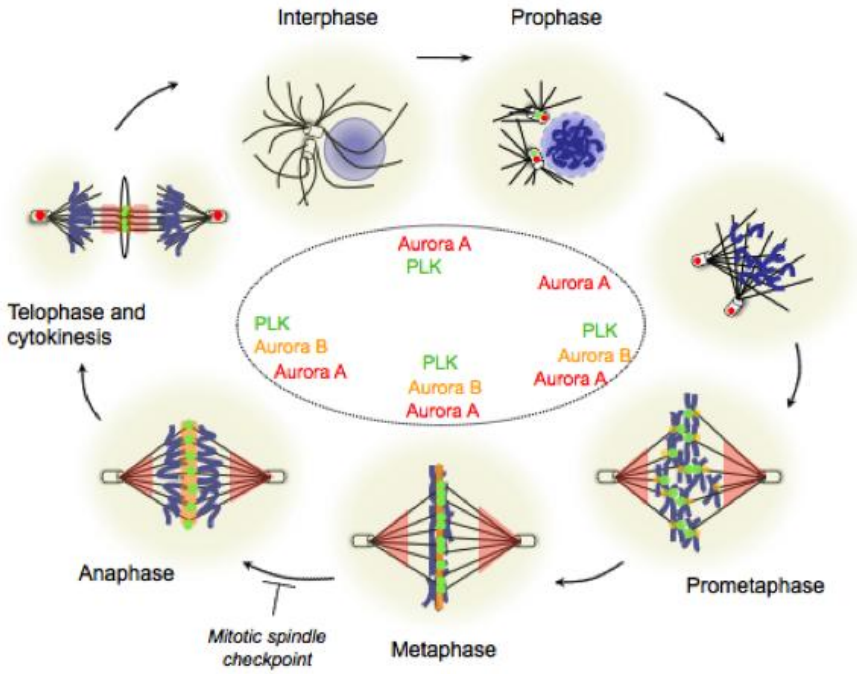


Table 27. The localization of mitotic kinases during mitosis. Adapted from Jackson *et al.*, 2007

These proteins do not act alone, but rather they operate in conjunction with other proteins. Table 7 summarizes the (putative) substrate proteins through which the effects of these mitotic proteins are mediated. As shown, they have crucial roles in initiating, advancing and concluding the process of mitosis. They often act in concert, but also appear to perform unique functions without overlap.

Mitotic kinase	(Putative) substrate	Localization/events	Selected references
Aurora A	BRCA	Centrosome/G_2–M transition	(Ouchi et al., 2004)
	Eg5 (KSP)	Spindle/centrosome separation	(Giet et al., 1999)
	Lats2	Centrosome/cell cycle check point	(Toji et al., 2004)
	TPX2	Mitotic spindle/spindle assembly	(Giet et al., 2005)
	CENP-A	Centromere/chromosome alignment	(Kunitoku et al., 2003)
	TACC3	Mitotic spindle/centrosome maturation	(Kinoshita et al., 2005)
	p53	Centrosome	(Liu et al., 2004)
	ASAP	Mitotic spindle/cell cycle	(Venoux et al., 2008)
	Cdc25b	Centrosome/centrosome duplication	(Dutertre et al., 2004)
Aurora B	Histone H3	Chromatin modification	(Goto et al., 2002)
	CENP-A	Centromere/cytokinesis	(Zeitlin et al., 2001)
	INCENP	Centromere/spindle checkpoint	(Sessa et al., 2005)
	Survivin	Centromere/spindle checkpoint	(Wheatley et al., 2001)
	MCAK	Centromere/chromatid separation	(Andrews et al., 2004)
	Desmin	Cytokinesis	(Kawajiri et al., 2003)
	Myosin II	Cytokinesis	(Murata-Hori et al., 2000)
	Vimentin	Cytokinesis	(Goto et al., 2003)
	Dam1	Kinetochore/chromosome segregation	(Cheeseman et al., 2002)
	Ndc80	Kinetochore/chromosome segregation	(Akiyoshi et al., 2009)
	Septin	Cytokinesis	(Qi et al., 2005)
	Stathmin	Microtubule disassemble	(Gadea & Ruderman, 2006)
Polo-like kinase	Cdc25	Nucleus/commitment to (entry into) mitosis	(Roshak et al., 2000)
	APC/C	Chromatid segregation	(Eckerdt & Strebhardt, 2006)
	Cyclin B	Kinetochore/cell cycle	(Toyoshima-Morimoto et al., 2001)
	SCC1 cohesin	Sister chromatid cohesion/ Chromosome segregation	(Sumara et al., 2002)
	Myt1	Cell cycle	(Nakajima et al., 2003)
	B23	Spindle poles/centrosome duplication	(Zhang et al., 2004)
	Nlp	Centrosome/spindle formation	(Casenghi et al., 2003)

Table 7. Targets of mitotic-kinase inhibitors

In vitro and xenograft models show extensive mitotic arrest followed by cell death upon treatment with a mitotic kinase inhibitor. For example, as detected by histone H3 phosphoserine staining on a frozen section of a H460 xenograft model, BI-2536 induces mitotic arrest in more than 50% of cells within 24 hours after intravenous drug administration (Figure 28). This mitotic arrest is followed by apoptosis in the next 24 hours shown by tunnel assay (Steegmaier et al., 2007).

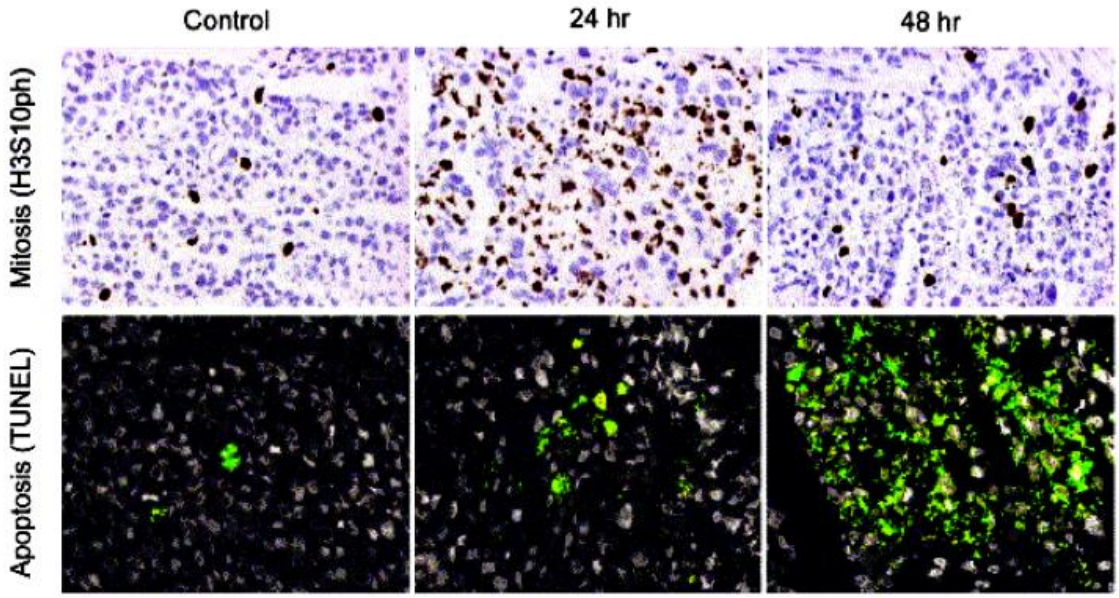


Figure 28. Mitotic kinase inhibitor, BI 2536, causes extensive mitotic arrest and apoptosis in H460 lung cancer xenograft model. Adapted from Steegmaier et al., 2007

It is not surprising that cells with rapid doubling times (such as *in vitro* and xenograft models) are vulnerable to mitotic poisons. The success of these preclinical models stands as evidence that mitotic kinase inhibitors are chemically well-designed and reach their targets. They inhibit the mitotic kinases they had been tailored for, and these evidently play a pivotal role in cell division. But can a group of drugs that is completely dependent on cell division be successful in treating slower growing human tumors?

In the following paragraphs I am going to present data about human cancer doubling times and human myeloid kinetics, and argue why these mitotic kinase inhibitors have been unsuccessful in the therapy of tumors in patients.

6.12. Human tumor doubling times

It is fortunate that the doubling times of human tumors are far greater than that of *in vitro* cell models and xenografts. We evaluated more than 100 studies, and 56 of them answered the question of the duration of human tumor doubling time. Our aim was to include frequently occurring tumor types with different origins and biology, such as lung cancer, colon cancer, hepatocellular cancer, breast cancer, prostate cancer, and melanomas (Table 8 - Table 15). This unbiased and extensive but not exhaustive compilation of literature data shows that the median doubling time of all tumors is approximately 147 days. Human tumor doubling times in these studies were calculated either by imaging techniques or by tumor marker levels. Similar results were obtained from studies that have looked at ^{3H}thymidine [^{3H}TdR] labeling or flash doubling times (Figure 29, Table 16) (Livingston *et al.*, 1974; Wolberg & Ansfield, 1971). The basis of the percent-label technique is that cells actively synthesizing DNA incorporate ^{3H}thymidine (^{3H}TdR). Shortly after flash labeling with the ^{3H}TdR radiotracer, a specimen is fixed, stained, and an autoradiograph prepared. For each sample the proportion of nucleated cells that have incorporated the label into their nuclei is determined. This labeling index (LI) represents the ratio of labeled cells or cells in DNA synthesis to total cells of a defined morphologic type – such as tumor cells. Thus the flash labeling index (the proportion of cells in a population which incorporates ^{3H}TdR at a point in time) is a measure of proliferative activity. Wolderg and Ansfield determined the percentage of cells labeled with ^{3H}thymidine in 565 tumor samples of different origin (Figure 29). They found that the percentage of cells labeled during the 24-hr incubation was 2.9. Based on their own data and literature search, Livingstone et al. concluded that the median labeling index of human melanomas ranges between 3.3 and 3.6 regardless of the method used to study proliferative activity (Table 16). It is evident that if only a few percent of cells (less than 5%) are undergoing DNA synthesis at any one time, the tumor doubling time has to be considerably longer than that of our *in vitro* and xenograft models.

Lung cancer: tumor doubling times	
(Quint et al., 2008)	
Adenocarcinoma	181 days (median)
Squamous-cell carcinoma	139 days (median)
Miscellaneous	137 days (median)
Metastasis	111 days (median)
(El Sharouni et al., 2003)	
NSCLC	29 days (median)
(Winer-Muram et al., 2002)	
Stage I lung cancer	181 days (median)
(Sato et al., 1999)	
Adenocarcinoma	196 days (mean)
(Arai et al., 1994)	
Adenocarcinoma	222 days (mean)
Squamous-cell carcinoma	115 days (mean)
Large-cell carcinoma	67 days (mean)
Small-cell carcinoma	86 days (mean)
Others	225 days (mean)
(Usuda et al., 1994)	
Adenocarcinoma	163 days (mean)
Squamous-cell carcinoma	80 days (mean)
Large-cell carcinoma	67 days (mean)
Small-cell carcinoma	69 days (mean)
(Filderman et al., 1986)	
Adenocarcinoma	180 days (mean)
Squamous-cell carcinoma	100 days (mean)
Large-cell carcinoma	100 days (mean)
Small-cell carcinoma	30 days (mean)
(Geddes, 1979)	
Adenocarcinoma	161 days (mean)
Squamous-cell carcinoma	88 days (mean)
Large-cell carcinoma	86 days (mean)
Small-cell carcinoma	29 days (mean)
(Fujimura S, 1979)	
Adenocarcinoma	116 days (mean)
Squamous-cell carcinoma	94 days (mean)
Large-cell carcinoma	71 days (mean)
Small-cell carcinoma	71 days (mean)
Range	**29–225 days**

Table 8. Lung cancer: tumor-doubling time

Colon cancer: doubling time of serum CEA	
(Yamamoto et al., 2004)	
Proximal colon cancer	71 days (average)
Distal colon cancer	31 days (average)
Rectal cancer	73 days (average)
(Ito et al., 2002)	
Colon cancer	68 days (median)
(Takesue et al., 1998)	
Recurrent colon cancer	85 days (mean)
(Takahashi et al., 1986)	
Colon cancer liver metastasis	57 days (mean)
(Umehara et al., 1993)	
Recurrent colorectal cancer	60 days (mean)
Liver metastasis	31 days (mean)
Lung metastasis	78 days (mean)
Peritoneal carcinomatosis	18 days (mean)
Local recurrence	153 days (mean)
Range	**18–153 days**

Table 9. Colon cancer: doubling time of serum CEA

Colon cancer: tumor-doubling times	
(Umetani et al., 2000)	
Non pedunculated colorectal carcinomas	6.8 months (mean)
(Matsui et al., 2000)	
Colorectal carcinoma mucosa	31 months (average)
Colorectal carcinoma submucosa	25 months (average)
Colorectal carcinoma muscularis propria	13 months (average)
Colorectal carcinoma serosa	13 months (average)
(Finlay et al., 1988)	
Overt liver metastasis	155 days (mean)
Occult liver metastasis	86 days (mean)
(Tada et al., 1984)	
Carcinomas	92–1032 days
Adenomatous polypus	146–398 days
(Bolin et al., 1983)	
Colon and rectal carcinoma	130 days (median)
Range	**86 days–31 months**

Table 10. Colon cancer: tumor-doubling times

Hepatocellular carcinoma: tumor-doubling times	
(Tezuka et al., 2007)	
Locally recurrent hepatocellular carcinoma	69 days (median)
(Chang et al., 2005)	
Hepatocellular carcinoma	112 days (mean)
(Wang et al., 2005)	
Hepatocellular carcinoma	104 days (mean)
(Taouli et al., 2005)	
Hepatocellular carcinoma	127 days (mean)
(Saftoiu et al., 2004)	
Hepatocellular carcinoma	140 days (mean)
(Kubota et al., 2003)	
Hepatocellular carcinoma	114 days (mean)
(Ebara et al., 1998)	
Hepatocellular carcinoma with cirrhosis	195 days (mean)
(Matsuhashi et al., 1996)	
Alcoholic	78 days (average)
Non-alcoholic	142 days (average)
(Sadek et al., 1995)	
Volume	9 weeks (average)
Diameter	29 weeks (average)
(Okada et al., 1993)	
Recurrent hepatocellular carcinoma	112 days (mean)
(Barbara et al., 1992)	
Hepatocellular carcinoma	171 days (median)
(Okazaki et al., 1989)	
Hepatocellular carcinoma	102 days (mean)
(Sheu et al., 1985)	
Hepatocellular carcinoma	117 days (median)
(Yoshino, 1983)	
Hepatocellular carcinoma	119 days (mean)
Range	**63–203 days**

Table 11. Hepatocellular carcinoma: tumor-doubling times

Breast cancer: tumor-doubling times	
(Tilanus-Linthorst et al., 2005)	
BRCA1/2 carrier	45 days (mean)
BRCA1/2 wild type	84 days (mean)
(Conant et al., 1994)	
Breast cancer	267 days (median)
(Peer et al., 1993)	
<50 years	80 days (median)
50–70 years	157 days (median)
>70 years	188 days (median)
(Arnerlov et al., 1992)	
Breast cancer	9 months (median)
(Kuroishi et al., 1990)	
Breast cancer	174 (geometric mean)
(Semiglazov et al., 1989)	
Breast cancer	94 days (average)
(Spratt et al., 1986)	
Carcinoma *in situ*	94 days (average)
Negative axillary nodes	75 days (average)
Positive axillary nodes	56 days (average)
(Koscielny et al., 1985)	
Primary tumor	7 months (median)
Metastasis	2.5 months (median)
(von Fournier et al., 1980)	
Breast cancer	212 days (average)
(Heuser et al., 1979)	
Breast cancer	325 days (mean)
(Spratt et al., 1977)	
Breast cancer	117 days (mean)
(Lundgren, 1977)	
Breast cancer	211 days (average)
Range	**45–325 days**

Table 12. Breast cancer: tumor-doubling times

Prostate cancer: doubling-time of serum PSA	
(Kawa et al., 2002)	
Prostate cancer	36.3 months (median)
(Choo et al., 2001)	
Prostate cancer	5.1 years (median)
(Arai et al., 2001)	
Prostate cancer	36.7 months
(Egawa et al., 2000)	
Prostate cancer	25.8 months (median)
(Egawa et al., 1999)	
Prostate cancer	35.7 months (median)
(Gerber et al., 1998)	
Prostate cancer	55.7 months (median)
(Nakata et al., 1998)	
Prostate cancer	23 months (average)
(Pruthi et al., 1997)	
Patients who failed to benefit from radical prostatectomy	284 days (median)
(Hanks et al., 1996)	
Prostate cancer	31 months (median)
(Yu et al., 1995)	
Patients whose cancer relapsed	277 days (mean)
(Lee et al., 1995)	
Prostate cancer	38 months (median)
(Pollack et al., 1994)	
Post radiotherapy	13.5 months (average)
(Schmid et al., 1994)	
Tumor confined to the prostate	4 years (median)
(Carter et al., 1992)	
Local/regional Advanced/metastatic	3.0 years (median) 2.0 years (median)
(Stamey & Kabalin, 1989)	
Stages A and B	At least 2 years
Range	**284 days–55.7 months**

Table 13. Prostate cancer: doubling-time of serum PSA

Prostate cancer: tumor doubling times	
(Berges et al., 1995)	
High-grade intraepithelial prostate cancer	154 days
Localized prostate cancer—low Gleason sum	577 days
Localized prostate cancer—high Gleason sum	495 days
No prior hormone therapy—metastases to lymph nodes	33 days
No prior hormone therapy—metastases to bones	54 days
Prior hormone therapy—metastases to sites other than bones	126 days
Prior hormone therapy—metastases to bones	94 days
Range	**33–577 days**

Table 14. Prostate cancer: doubling time of serum PSA

Melanoma: tumor doubling times	
(Lee et al., 2009)	
Pulmonary metastasis	61 days (median)
(Carlson, 2003)	
Primary	94 days (median)
Metastatic	33 days (median)
(Eskelin et al., 2000)	
Metastatic uveal melanoma—treated but no response	225 days (median)
Untreated metastasis	63 days (median)
(Char et al., 1997)	
Uveal melanomas	1.4 years (median)
(Schotterl & Paul, 1988)	
Pulmonary metastasis—males	35 days (mean)
Pulmonary metastasis—females	36 days
(Augsburger et al., 1984)	
Posterior uveal melanomas—nine mixed cells	128 days (mean)
Posterior uveal melanomas—eight spindle cells	291 days (mean)
Range	**33 days–1.4 years**

Table 15. Melanoma: tumor doubling times

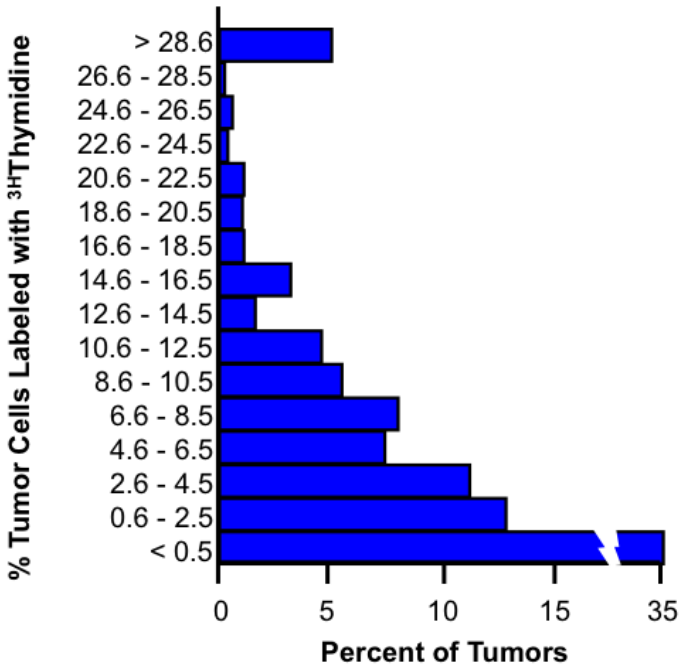


Figure 29. Percentage of tumor cells labeled with ^{3H} thymidine. Adapted from Wolberg and Ansfield, 1971

Flash Labeling Index (Percentage) Human Melanoma
(Livingston *et al.*, 1974)

Reference (Year)	**Method [Number of Patients]**	**Median Labeling Index**
Terz et al (1971)	*In vivo*, systemic TdR-^{3}H [1]	23
Muggia & De Vita (1972)	*In vivo*, local TdR-^{3}H [1]	8
Wolberg & Brown (1962)	*In vitro*, tissue slices - 24h [2]	0.55
Shirakawa et al (1970)	*In vivo*, systemic TdR-^{3}H [4]	5
Titus & Shorter (1965)	*In vitro*, tissue slices [1]	3.3
Sky-Peck (1971)	*In vitro*, tissue slices [3]	2.0
All above	**All above [12]**	**3.6 (0 - 23)**
Livingston et al.	***In vitro*, cell suspension [25]**	**3.3 (0.5 - 12)**

Table 16. Flash labeling index of human melanoma. Adapted from Livingston et al, 1974

Longer doubling times of human tumors do not come with extended cell cycle duration. In most cases, the duration of cell cycle has been shown to range between 15 and 125 hours. S-phase, a discrete period of interphase that is dedicated for DNA synthesis, requires approximately 9.5 to 24 hours (Tubiana M, 1976). Studies have demonstrated that the amount (and fraction) of time spent in mitosis is only a small percentage of the total doubling time (ranging only between 0.5 and 2.5 hr) (Baserga, 1965). This observation is largely self-evident given that arrest in mitosis is intolerable to cells (Rixe & Fojo, 2007; Smith & Martin, 1973).

Cell Cycle Kinetics of Solid Tumors
(Tubiana M, 1976)

Reference (Year)	**Duration of the Cell Cycle in Humans Solid Tumors of Various Histologic Types (Percent Labeled Mitoses Technique)**
Frindel et al (1968)	97, 51.5, 27.5, 48, 49.8
Bennington (1969)	15.5, 14.9
Young and De Vita (1970)	42, 82, 74
Shirakawa et al (1970)	120, 144
Weinstein and Frost (1970)	217
Terz et al (1971)	44.5, 31, 14, 25.5, 26
Estevez et al (1972)	37, 30, 48, 30, 38, 96, 48
Muggia et al (1972)	64
Peckham and Steel (1973)	59
Terz and Curutchet (1974)	18, 19, 19.2, 120
Bresciani et al (1974)	82, 50, 67, 53, 58
Malaise et al	24, 33, 48, 42
Duration of cell cycle: 15 - 125 hours (Modal: 48 hours)	
Duration of S phase: 9.5 - 24 hours (Modal: 16 hours)	

Table 17. Cell cycle kinetics of solid tumors. Adapted from Tubiana M, 1976

These examples clearly demonstrate that the fraction of time spent in mitosis and in turn the fraction of cells expressing the desired “mitotic specific target” are small. Furthermore, mitotic figures are rarely present in patient samples and are also not observed if the tumor is examined shortly after treatment with a microtubule-targeting agent. For example, Denduluri et al. treated breast cancer patients with ixabepilone, an epothilone analog that stabilizes microtubules and lead to mitotic arrest *in vitro* and in xenograft models (Denduluri et al., 2007). Tumor biopsies were taken before and after

treatment, and stained with an antibody that recognizes acetylated ∝-tubulin, a marker for stable microtubules (Figure 30). As can be seen, despite extensive microtubule stabilization evidenced by increased levels of acetylated ∝-tubulin (brown) following drug treatment, mitotic figures are rarely seen. This finding also underscores the long doubling times of human tumors, and stands in stark contrast to the xenograft image shown in Figure 28.

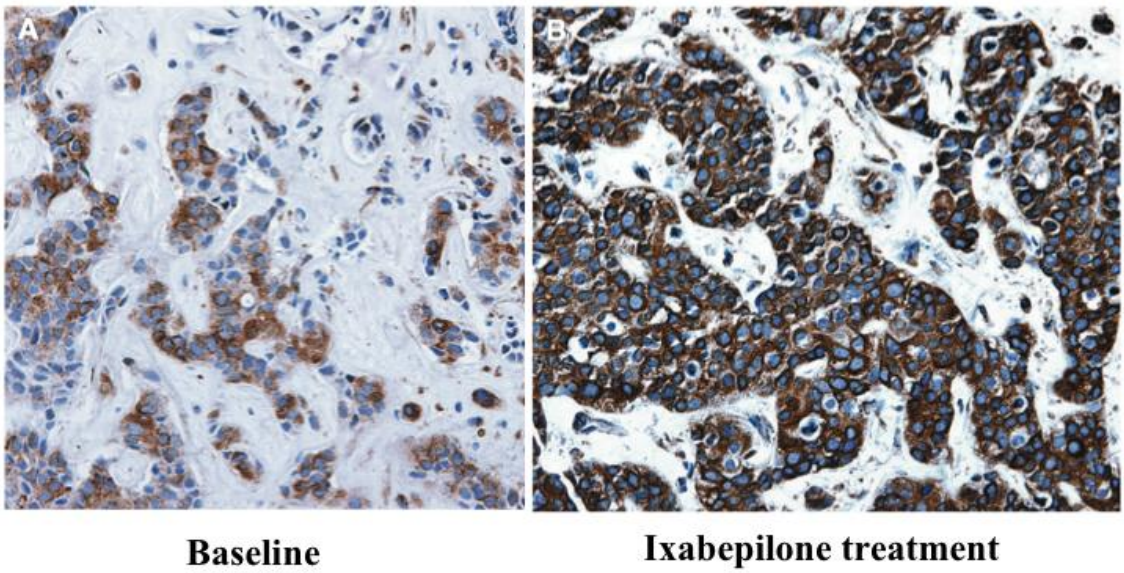


Figure 30. Despite evidence of microtubule stabilization following treatment with a microtubule-targeting agent, there is no evidence of mitotic arrest in human tumors. Adapted from Denduluri et al., 2007

6.13. Myeloid kinetics

While tumor cells have long doubling time in humans, there is one organ in which cells are undergoing very rapid turnover. Remarkably, at any one time 28% of total marrow neutrophils are in mitosis and total marrow neutrophil production amounts to 0.87 X 10^9 cells/kg/day, a rate that is equal to 6.1 X 10^{10} cells/day in a 70 kg subject (Table 18). As a comparison, the latter means that the normal marrow output in one day is an amount similar to the number of cancer cells in seven to eight two-centimeter tumors. It is not surprising that any drug that targets cell division leads to neutropenia.

Mean total marrow neutrophils*	7.70 ± 1.20 × 10^9 cells/kg
The mean number of normoblasts*	5.07 ± 0.84 × 10^9 cells/kg
Neutrophil:Erythroid ratio*	1.52 ± 0.07[1]
Post-mitotic pool*	5.59 ± 0.90 × 10^9 cells/kg (73%)
Mitotic pool*	2.11 ± 0.36 × 10^9 cells/kg (27%)
Neutrophil postmitotic transit time*	6.60 ± 0.03 days[1]
Rate of marrow neutrophil production*	0.85 × 10^9 cells/kg per day
Seven 2 cm tumors	5.6 X 10^{10} cells
Neutrophil turnover*	1.62 ± 0.46 × 10^9 cells/kg per day

*Data from 13 normal human bone marrows. ‡Metamyelocytes, bands and segmented neutrophils. §Promyelocytes and myelocytes

Table 18. Neutrophil cellularity and kinetics in humans. Adapted from Dancey *et al.*, 1976

6.14. Limitations of therapies targeting mitotic kinases

It is self-evident that for a targeted therapy to be effective, the target must be present. While in an in vitro or a xenograft model with a doubling time of 24 to 48 hours a large fraction of cells will prove vulnerable to a therapy that targets a mitosis specific protein, it is not surprising that in a patient with a tumor doubling time of more than 100 days, only a small, insignificant fraction of cells will be vulnerable to a drug aimed at a target that is expressed only transiently. Indeed, activity in preclinical models has been observed only with frequent administration – twice weekly better than weekly – reflecting the fact that even in these models with rapid doubling times, a fraction of cells not in G2/M are refractory to therapy and can repopulate the rapidly dividing tumor mass unless frequent administration is used (Steegmaier *et al.*, 2007).

The poor activity of mitotic kinase inhibitors is shown in Table 19. Infrequent responses have been observed clinically in cases where a kinase other than a mitotic kinase has accidentally emerged as a target. With MK-0457 (VX-680), for example, activity has been reported in patients with imatinib-resistant CML harboring the T315I mutation, a mutant form of Bcr-Abl which studies showed MK-0457 binds with very high affinity (K_d = 5 nM) by binding in a mode that accommodates the substitution of

the bulkier isoleucine for threonine at residue 315 (Giles et al., 2007). “Activity characterized as stable disease” has also been reported. For example, XL228, a multi-targeted protein kinase inhibitor targeting not only Aurora A, but also inhibiting IGF1R, FGFR1-3, ABL, ALK, and the SRC family of kinases, led to “stable disease” in 32% of cases in the escalating portion of the phase I study. In the same trial “stable disease” was shown in 41% of cases treated with the maximum tolerable dose including 5 of 9 (56%) non-small cell lung cancer patients (D. C. Smith, 2010). Given the large investment needed to develop a drug, it is not surprising that some have tried to rescue these drugs from their ultimate fate by assigning “stable disease” as a measure of activity. While we doubt any “targeted agent” can “arrest” a cancer cell for weeks to months in any phase of the cell cycle other than G_0, it is doubtful that arresting a cell in mitosis for other than a brief period can ever happen. An arrest in mitosis sufficient to qualify as a meaningful “stable disease” – several months – will never happen. There is a reason cells find mitotic arrest intolerable: transcription from packed chromosomes is impossible (Andersson et al., 1982). We believe the observation of “stable disease” is rather due to the tumor biology than the response for drug treatment.

The most frequently occurring dose limiting toxicity of mitotic kinase inhibitors is febrile neutropenia. The rapid turn over of neutrophils in the bone marrow makes them susceptible for mitotic kinase inhibitors. The occurrence of febrile neutropenia stands as evidence that this class of agents has excellent pharmacodynamic and pharmacokinetic characteristics, and that poor drug bioavailability cannot be held responsible for the lack of drug response.

Compound	Company	Comment
Aurora inhibitors		
MK-0457 (VX-680)	Vertex/Merck	Response in 3 patients with imatinib-resistant T315I CML, suspended due to cardiac safety (Giles *et al.*, 2007); SD in 3/16 patients[2]
AZD 1152	Astra Zeneca	No activity reported
PHA 680632	Nerviano	No activity reported
PHA 739358	Nerviano	SD in 7/42 patients[3]
MLN8054	Millenium	SD in 3/43 patients[4]
MLN8237	Millenium	SD in 5/23 patients; 1 case of PR and 8/65 patients with SD[5]
R763	Merck/Serono	PD in 2/15 patients[6]
AT9283	Astex	HR in 2/29 patients;[7] SD in 3/22 patients;[8] 1 case of PR and 4/33 patients with SD[9]
SNS-314	Sunesis	SD in 6/32 patients[10]
SU 6668	Sugen/Pfizer	SD in 4/35 patients;[11] SD in 3/19 patients[12]
ENMD-2076	EntreMed	SD in 4/14 patients[13]
BI 811283	Boehringer Ingelheim	SD in 33.3% of 57 patients;[14] SD in 29% of 52 patients[15]
CYC116	Cyclacel	One study ongoing, one study terminated
ENMD 981693	Entremed/Miikana	Ongoing
MKC 1693	Entremed/Miikana	Ongoing
Polo-like kinase inhibitors		
ON01910	Onconova	SD in 1/23 patients;[16] SD in 2/5 patients;[17] no response[18]
BI-2536	Boehringer	PR in 4/95 patients;[19] PR in 2/33 patients;[20] SD in 7/23 patients[21]
GSK-461364	GlaxoSmithKline	SD in 2/27 patients[22]
HMN-214	Nippon Shinyaku	No activity;[23] MR and SD;[24] 1 TR and 2 SD out of 33 patients[25]
BI-6727	Boehringer	3 PR, 48% SD and PFS in 6 out of 65 patients[26]
Kinesin spindle protein inhibitors		
Ispinesib (SB-715992)	Cytokinetics	SD in 4/42 patients;[27] SD in 3/27 patients;[28] unconfirmed MR in 3/24 patients;[29] SD in 2 and MR in 1 out of 27 patients;[30] SD in 7/19 patients;[31] 3 PR and SD in 4/16 patients[32]
SB-743921	Cytokinetics	1 PR and SD in 1/32 patients;[33] PR in 2/51 patients with lymphoma[34]
MK-0731	MRK	SD in 16/35 patients[35]
ARRY-520	Array Biopharma	PSD in 3/34 patients;[36] PR in 1/13 patients[37]

Abbreviations: CML, chronic myeloid leukemia; HR, hematologic remission; MR, minor response; PD, progressive disease; PFS, progression-free survival; PR, partial response; PSD, prolonged stable disease; SD, stable disease; TR, transient response.

Table 19. Agents that disrupt mitosis: limited activity to date

6.15. Interphase cells are the target of microtubule targeting agents

The clinical failure of mitotic kinase inhibitors ratified for us the mechanism of action of microtubule-targeting agents in human tumors. While mitotic arrest can explain the activity of microtubule-targeting agents in cells *in vitro*, in xenograft models, and in cells in human tumors that happen to be in mitosis at the time a microtubule-targeting agent is administered, it cannot explain the success of these drugs in the clinic. In addition to its role in mitosis, the microtubule cytoskeleton is essential for intracellular trafficking. Table 20 presents a partial list of proteins that either associate with or traffic on microtubules and that are considered crucial to most cells, but especially cancer cells—the list includes many crucial oncoproteins including p53 (Giannakakou P et al. Nat Cell Biol. 2000; 2:709-17), BRCA1 (Hsu LC and White RL. Proc Natl Acad Sci U S A. 1998; 95:12983-8; Lotti LV et al. Genes Chromosomes Cancer. 2002; 35:193-203), Rb (Roth DM et al. *Traffic 2007;* **8**:673–686). and androgen receptor (Zhu ML et al. Cancer Res.2010; 70:7992). For these proteins, an association with microtubules has been demonstrated by immunohistochemistry or in immunoprecipitation or pull-down experiments. Trafficking on microtubules has been inferred from an effect of treatment with a microtubule-targeting agent or over-expression of dynamitin, a protein that interferes with dynein-mediated trafficking (Roth et al., 2007). We would argue that interfering with the ability of these crucial proteins to traffic on microtubules would be disruptive to a cancer cell and that such a disruption could lead to cell death.

Protein/Gene	**Function**	**Selected references**
p53	Role in genomic integrity and apoptosis	(Giannakakou *et al.*, 2000)
c-Myc	Roles in proliferation and tumorigenesis	(Alexandrova et al., 1995)
APC protein	Regulates cell cycle, degrades ß-catenin in Wnt signaling pathway	(Jimbo et al., 2002; Munemitsu et al., 1994)
BRCA1	Maintains genomic integrity; controls cell growth	(Hsu & White, 1998; Lotti *et al.*, 2002)
RB	Role in cell cycle, tumor suppressor protein	(Roth *et al.*, 2007)
Smad2, 3 and 4	Signal transducers and transcriptional modulators, signaling effectors of TGF-ß	(Dong et al., 2000)
Glucocorticoid	Binds/transports glucocorticoids	(Akner et al., 1990;

receptor		Akner et al., 1995; Harrell et al., 2004)
Aryl hydrocarbon receptor	Cytochrome P450 (CYP) induction	(Vrzal et al., 2008)
Androgen receptor	Binds/transports androgens	(Zhu et al.)
Retinoic acid receptors	Nuclear receptors with diverse functions	(Dvorak et al., 2007)
GLUT4	Insulin-responsive glucose transporter	(Fletcher et al., 2000; Guilherme et al., 2000)
HSP 90 - HSP 70	Heat shock protein / important chaperone	(Marchesi & Ngo, 1993; Redmond *et al.*, 1989)
PTHrP	Apoptosis, proliferation and hypercalcemia	(Lam et al., 2002)
MIZ-1	Myc-interacting zinc finger protein, regulates c-myc	(Peukert et al., 1997; Ziegelbauer et al., 2001)
Elk-1	ETS domain transcription factor, stimulates transcription	(Demir et al., 2009)
Runx2	Regulator of cell growth and differentiation	(Pockwinse et al., 2006)
PP2A	Phosphatase activity, cell growth and transformation	(Sontag et al., 1995)
MID1, MID2	Ubiquitin ligase; Regulates MT function, mutated in Opitz syndrome	(Perry et al., 1999; Schweiger et al., 1999)
Src	Regulation of cell growth, axonal guidance	(Wu et al., 2008)
NMDA receptor	Controlling synaptic plasticity and memory function	(Setou et al., 2000; van Rossum et al., 1999)
CARD6	Interacts with receptor-interacting protein kinases and modulate signal transduction pathways	(Dufner et al., 2006)
ER-alpha	DNA binding transcription factor	(Manavathi et al., 2006)
P120-catenin	Functions in adhesion between cells and signal transduction	(Chen *et al.*, 2003; Roczniak-Ferguson & Reynolds, 2003)
Cyclophilin-A	Accelerates the folding of proteins	(Galigniana et al., 2004)
PKC	Protein kinase, mitogenic signaling	(Garcia-Rocha et al., 1997)
Parkin	Mediates proteasomal degradation as part of an E3 ligase complex	(Ren et al., 2003)
c-CBL	Cell signalling and protein	(Teckchandani et al.,

	ubiquitination	2005)
JWA	Pro-apoptotic molecule	(Chen Hairong, 2004)
PASK	Regulates intracellular signaling pathways; kinase	(Tsutsumi et al., 2008)
N-cadherin	Cell-cell adhesion, cell growth, migration, differentiation	(Mary et al., 2002)

Table 20. Partial list of proteins that traffic in or associate with microtubules

Impaired intracellular trafficking is one, but not the only effect of microtubule-targeting agents in interphase cells. Other effects include alterations of cell signaling and trafficking (Etienne-Manneville; Ganansia-Leymarie *et al.*, 2003; Herbst & Khuri, 2003) including reactive oxygen species (ROS) signaling (Jang et al., 2008), slowing of cell-cycle progression (Pasquier et al., 2004), alterations of cell migration and invasiveness (Tran et al., 2009), and vascular disruption (Kanthou & Tozer, 2007). Among these effects, vascular disruption characterized by rapid changes in the tumor vasculature leading to infarction and necrosis has received the most attention. The facts that vascular endothelial cells are easily accessible to chemotherapeutic drugs, and because they are not transformed are genetically stable, made them attractive targets. Reports of vascular disruption with low concentrations of some microtubule targeting agents, such as the combretastatins, led investigators to refer to this class of microtubule-targeting agents as vascular-disrupting agents. Although the mechanism of action of vascular-disrupting agents is not fully understood, exposure of endothelial cells to microtubule-targeting agents *in vitro* leads to numerous changes including the disruption of cell–cell adhesions, malformations of focal adhesions, induced action-myosin contractility, accumulation of actin stress fibers, and phosphorylation of myosin light chain (Pasquier et al., 2007). *In vivo,* the administration of vascular-disrupting agents is followed by rapid collapse and increased permeability of the established tumor with the center of the tumor more susceptible than the outer rim (Dark et al., 1997). This rapid antivascular effect cannot be explained by inhibition of mitosis but rather by the morphological and functional changes that occur in endothelial cells. These changes are due to disruption of the function of interphase microtubules.

Another strong evidence that microtubule targeting agents can induce cell death independent of mitosis can also be found in the primary toxicity of microtubule targeting agents – neurotoxicity – that is seen in as many as 70 to 90% of patients treated (DeAngelis et al., 1991; Forsyth et al., 1997; Postma et al., 1995). Peripheral neuropathy secondary to microtubule-targeting agents is usually bilateral, and preferentially involves sensory neurons over thick myelinated nerves. It commonly affects the distal lower limbs first, and later involves the distal upper extremities in what is often described as a 'stocking–glove distribution', suggesting the longer the nerve is, the more vulnerable it is to damage (Mielke et al., 2006). This selectivity for long neurons argues that microtubule-dependent transport from the neural cell body down the axon is being compromised. Although a limited number of studies have biopsied neurons, fiber loss, axonal atrophy and secondary demyelination have been observed following paclitaxel treatment (Sahenk et al., 1994). Since neuronal cells rarely, if ever, divide in adults, in this instance microtubule targeting agents have obviously caused the death of non-dividing cells (Argyriou et al., 2008).

Why have microtubule-targeting agents succeeded in patients whereas to date success has eluded mitotic inhibitors? The steady presence and constant physiological role of their target is responsible for the overall success of microtubule-targeting agents compared to mitotic inhibitors. While mitotic inhibitors are effective only on a small fraction of the tumor mass (on dividing cells), microtubule-targeting agents target microtubules directly or by interacting with tubulin thus interfering with microtubule function in both dividing and non-dividing cells. Few targets can match the appeal that mitotic kinases have had. Scientifically exciting, involved in a crucial cellular function, without clear redundancy and eminently "druggable", the temptation was too great. The expectation was that they would be as good if not better than microtubule-targeting agents but without neurotoxicity. The disappointing clinical results – a lack of meaningful activity but with marked myelosuppression – indicate the drugs taken to the clinic were well designed and the target a valid one for rapidly dividing cells. However, as the data presented demonstrate, the majority of human tumors do not divide rapidly enough to be susceptible to such drugs and more importantly they divide much slower than vulnerable marrow elements. Due their disappointment, however, we now have

data that validate a paradigm for how microtubule-targeting agents kill cancer cells in patients. We conclude that microtubule-targeting agents obstruct proper cell separation in dividing cells, and also block cell trafficking in non-dividing cells. While the two mechanisms together result in the exposure of the whole tumor population, the latter phenomenon alone leads to an undesired side effect - neuropathy.

7. Conclusions

- The KB-3.1 cell line has wild-type p53.

- The KB-CP20 cell line harbors a frequently occurring mutation in the TP53 gene. This mutation, a missense mutation that changed amino acid 172 from valine to phenylalanine (V172F), alters the DNA-binding domain of the protein leading to a transcriptionally non-functional p53.

- The KB-OX cell lines harbor a unique mutant p53 protein. A deletion of a single nucleotide in the sequence encoding for amino acid 382 alters the nuclear localization signal III and results in a frame-shift mutation leading to a protein designated as $p53^{420}$ that is 27 amino acids longer than wt p53.

- This mutant $p53^{420}$ is sequestered primarily in the cytoplasm, although it is able to tetramerize and bind to dynein, the negative motor protein of the microtubules.

- The reduced nuclear accumulation of $p53^{420}$ is not the consequence of an augmented nuclear export.

- The p53 nuclear localization signals II and III are not essential for nuclear translocation of the protein.

- The mutation in $p53^{420}$ confers a relative instability to the protein, although the levels of the protein in the oxaliplatin resistant cells are high.

- Although the $p53^{420}$ protein can bind importin the binding is impaired. The poor interaction of $p53^{420}$ with importin is the explanation for the retention of $p53^{420}$ in the cytoplasm.

- $p53^{420}$ cytoplasmic sequestration is advantageous for the cancer cells, because this mutant p53 retains the ability to transactivate p53-inducible genes such as p21.

- Like several (onco)proteins, p53 relies on microtubule trafficking to enter the nucleus. The administration of a microtubule-targeting agent impairs p53 nuclear accumulation.

- Microtubule-targeting agents are valuable drugs in the treatment of human solid tumors. Their efficacy was shown in preclinical models, and they are members of many first-line cancer treatment regimens.

- Mitotic kinase inhibitors have shown activity in preclinical models, but to date have failed to provide benefit to patients in clinical trials.

- The doubling times of immortalized cancer cells are much shorter than that of human cancers.

- Human tumor doubling-times are longer than that of neutrophils in the bone marrow.

- The serious side effect of the mitotic kinase inhibitors (febrile neutropenia) proves that these drugs are well-designed, target rapidly dividing cells, and have excellent bioavailability.

- The clinical failure of mitotic kinase inhibitors stands as an evidence that microtubule-targeting agents target cells not only in mitosis but also in interphase.

- Neurotoxicity stands as strong evidence that microtubule-targeting agents target non-dividing cells too.

8. Summary

Intracellular trafficking of p53 on microtubules is followed by its nuclear traslocation that is mediated via nuclear localization signal-I (NLS-I). By screening the KB-3.1 human cervical carcinoma cell line and its drug resistant sublines, we found a unique p53 mutation in the oxaliplatin resistant cell lines. This mutation resulted in a frame-shift and a protein of 420 amino acids ($p53^{420}$). Additional studies showed that $p53^{420}$ was sequestered in the cytoplasm. We demonstrated that $p53^{420}$ was able to tetramerize, bind to dynein, and that cytoplasmic sequestration of $p53^{420}$ is due to impaired nuclear import. We also showed that $p53^{420}$ was able to trans-activate a target gene. This behavior of $p53^{420}$ underscores the necessity of its cytoplasmic sequestration so that it can contribute to the survival advantage of the cancer cell that harbors it. In addition, we demonstrated that neither NLS II nor NLS III plays a role in p53 nuclear translocation and we suggest dropping nomenclature that assigns a nuclear transport function to these regions of the protein. Like p53, many other proteins traffic on the microtubules from the site of production to the site of function. Impaired trafficking, for example due to treatment with a microtubule-targeting agent, seriously interferes with the function of these proteins and in turn the viability of the cell. Based on preclinical studies, microtubule-targeting agents were proposed to be clinically successful by causing mitotic arrest. Indeed it was the success of microtubule-targeting agents, and the attribution of this to mitotic arrest, that led to the search for more mitosis-specific drugs, and to the development of mitosis kinase inhibitors. The expectation was that mitotic kinase inhibitors would be as good as if not better than microtubule-targeting agents but without neurotoxicity. Following responses seen in preclinical models, clinical trails did not deliver on this expectation. In their disappointment, however, we now have data that validate a paradigm for how microtubule targeting agents kill cancer cells in patients – principally by interfering with the function of interphase microtubules, for example intracellular trafficking – not by disrupting mitosis. We believe that the steady presence and constant physiological role of microtubules are responsible for the overall success of microtubule-targeting agents. While mitosis-specific inhibitors are effective on only a small fraction of the tumor mass (dividing cells), microtubule-targeting agents target microtubules directly or by associating with its tubulin subunits, and thus interfere with a protein that has crucial roles in both mitotic and non-mitotic cells.

9. Összefoglalás

A p53 fehérje a mikrotubulus segítségével eljut a sejtmag közelébe, ahol a fehérje sejtmagba történő belépését a magi lokalizációs szignál-I (MLSZ-I) segíti elő. A KB-3.1 humán méhnyakrák sejtvonal és ennak a gyógyszer rezisztens alvonalainak vizsgálata során egy ritkán előforduló p53 mutációt találtunk az oxaliplatin rezisztens sejtvonalban. Ennek a ”frame-shift” mutációnak a következménye egy 420 aminósavat tartalmazó p53 fehérje ($p53^{420}$). Kísérleteink igazolták, hogy a $p53^{420}$ a sejtplazmában szekvesztrálódik. Vizsgálatainkkal bizonyítottuk, hogy $p53^{420}$ tetramert alkot, dyneinhez kötődik, és hogy a sejtplazmába történö szekvesztrálása az elégtelen sejtmagi belépésének a következménye. Emellett kimutattuk, hogy a $p53^{420}$ képes egy célgén transzaktiválására. Ez utóbbi tulajdonság igazolja a sejtplazmai szekvesztráció szükségességét, ugyanis ennek a fehérjének a sejmagi kizárása hozzájárul a daganatos sejtek túléléséhez. További kísérleteinkkel bizonyítottuk, hogy a második és a harmadik MLSZ nem játszanak szerepet a p53 sejtmagba való belépésében. Ezért javasoljuk ezen régiók „MLSZ”-ként való elnevezésének az elhagyását. A p53 fehérjéhez hasonlóan más fehérjék is a mikrotubulus segítségével jutnak el a termelődés helyétől a működés helyére. Ennek a mozgásnak a meggátolása (például a mikrotubulusokra ható gyógyszerekkel) megakadályozza ezen fehérjék működését, és ezáltal a sejtek életképességét. Laboratóriumi eredmények alapján a mikrotubulusokra ható szerek klinikai sikerét a sejtosztódás megakadályozásának tulajdonították. A klinikai sikerek és ennek a hátterében álló feltételezett hatások vezettek a sejtosztódásra ható gyógyszerek kutatásához, és a mitóziskináz-gátlók kifejlesztéséhez. A várakozások szerint ezek a szerek ugyanolyan hatásosak -ha nem hatásosabbak- lettek volna mint a mikrotubulusra ható szerek mellékhatások nélkül. Az ígéretes laboratóriumi eredmények nem váltották be a hozzájuk fűzött reményeket a klinikumban, ugyanakkor hozzájárultak egy új hatásmechanizmus feltételezéséhez, miszerint a mikrotubulusra ható szerek a nyugvó sejtek fehérjemozgásának meggátolásán át fejtik ki hatásukat, és nem pedig a sejtosztódás megakadályozásán keresztül. Úgy véljük, hogy a mikrotubulusok állandó jelenléte és fiziológiás szerepe a felelős a rájuk ható gyógyszerek klinikai sikeréért. Míg a mitóziskináz-gátlók csak a daganat egy elenyésző részére hatnak (az osztodó sejtekre), ezzel szemben a mikrotubulusra ható szerek a mikrotubulust célozzák, amelynek fontos szerepe van mind az osztodó, mind a nyugvó sejtek életében.

10. References

Akhmanova, A.Steinmetz, M. O. (2008). Tracking the ends: a dynamic protein network controls the fate of microtubule tips. *Nat Rev Mol Cell Biol* **9,** 309-22.

Akiyoshi, B., Nelson, C. R., Ranish, J. A. &Biggins, S. (2009). Analysis of Ipl1-mediated phosphorylation of the Ndc80 kinetochore protein in Saccharomyces cerevisiae. *Genetics* **183,** 1591-5.

Akner, G., Sundqvist, K. G., Denis, M., Wikstrom, A. C. &Gustafsson, J. A. (1990). Immunocytochemical localization of glucocorticoid receptor in human gingival fibroblasts and evidence for a colocalization of glucocorticoid receptor with cytoplasmic microtubules. *Eur J Cell Biol* **53,** 390-401.

Akner, G., Wikstrom, A. C. &Gustafsson, J. A. (1995). Subcellular distribution of the glucocorticoid receptor and evidence for its association with microtubules. *J Steroid Biochem Mol Biol* **52,** 1-16.

Alexandrova, N., Niklinski, J., Bliskovsky, V., Otterson, G. A., Blake, M., Kaye, F. J., *et al.* (1995). The N-terminal domain of c-Myc associates with alpha-tubulin and microtubules in vivo and in vitro. *Mol Cell Biol* **15,** 5188-95.

Andersson, K., Mahr, R., Bjorkroth, B. &Daneholt, B. (1982). Rapid reformation of the thick chromosome fiber upon completion of RNA synthesis at the Balbiani ring genes in Chironomus tentans. *Chromosoma* **87,** 33-48.

Andrews, P. D., Ovechkina, Y., Morrice, N., Wagenbach, M., Duncan, K., Wordeman, L., *et al.* (2004). Aurora B regulates MCAK at the mitotic centromere. *Dev Cell* **6,** 253-68.

Appella, E.Anderson, C. W. (2001). Post-translational modifications and activation of p53 by genotoxic stresses. *Eur J Biochem* **268,** 2764-72.

Arai, T., Kuroishi, T., Saito, Y., Kurita, Y., Naruke, T. &Kaneko, M. (1994). Tumor doubling time and prognosis in lung cancer patients: evaluation from chest films and clinical follow-up study. Japanese Lung Cancer Screening Research Group. *Jpn J Clin Oncol* **24,** 199-204.

Arai, Y., Egawa, S., Kuwao, S., Ogura, K. &Baba, S. (2001). The role of volume-weighted mean nuclear volume in predicting tumour biology and clinical behaviour in patients with prostate cancer undergoing watchful waiting. *BJU Int* **88,** 909-14.

Argyriou, A. A., Koltzenburg, M., Polychronopoulos, P., Papapetropoulos, S. &Kalofonos, H. P. (2008). Peripheral nerve damage associated with administration of taxanes in patients with cancer. *Crit Rev Oncol Hematol* **66,** 218-28.

Arnerlov, C., Emdin, S. O., Lundgren, B., Roos, G., Soderstrom, J., Bjersing, L., *et al.* (1992). Breast carcinoma growth rate described by mammographic doubling time and S-phase fraction. Correlations to clinical and histopathologic factors in a screened population. *Cancer* **70,** 1928-34.

Augsburger, J. J., Gonder, J. R., Amsel, J., Shields, J. A. &Donoso, L. A. (1984). Growth rates and doubling times of posterior uveal melanomas. *Ophthalmology* **91,** 1709-15.

Barbara, L., Benzi, G., Gaiani, S., Fusconi, F., Zironi, G., Siringo, S., *et al.* (1992). Natural history of small untreated hepatocellular carcinoma in cirrhosis: a multivariate analysis of prognostic factors of tumor growth rate and patient survival. *Hepatology* **16,** 132-7.

Baserga, R. (1965). The Relationship of the Cell Cycle to Tumor Growth and Control of Cell Division: A Review. *Cancer Res* **25,** 581-95.

Becker, K., Marchenko, N. D., Maurice, M. &Moll, U. M. (2007). Hyperubiquitylation of wild-type p53 contributes to cytoplasmic sequestration in neuroblastoma. *Cell Death Differ* **14,** 1350-60.

Beijnen, J. H. (1994). p53 selected as molecule of the year 1993. *Pharm World Sci* **16,** 1.

Berges, R. R., Vukanovic, J., Epstein, J. I., CarMichel, M., Cisek, L., Johnson, D. E., *et al.* (1995). Implication of cell kinetic changes during the progression of human prostatic cancer. *Clin Cancer Res* **1,** 473-80.

Bolin, S., Nilsson, E. &Sjodahl, R. (1983). Carcinoma of the colon and rectum--growth rate. *Ann Surg* **198,** 151-8.

Bosari, S., Viale, G., Bossi, P., Maggioni, M., Coggi, G., Murray, J. J., *et al.* (1994). Cytoplasmic accumulation of p53 protein: an independent prognostic indicator in colorectal adenocarcinomas. *J Natl Cancer Inst* **86,** 681-7.

Bosari, S., Viale, G., Roncalli, M., Graziani, D., Borsani, G., Lee, A. K., *et al.* (1995). p53 gene mutations, p53 protein accumulation and compartmentalization in colorectal adenocarcinoma. *Am J Pathol* **147,** 790-8.

Carlson, J. A. (2003). Tumor doubling time of cutaneous melanoma and its metastasis. *Am J Dermatopathol* **25,** 291-9.

Carter, H. B., Morrell, C. H., Pearson, J. D., Brant, L. J., Plato, C. C., Metter, E. J., *et al.* (1992). Estimation of prostatic growth using serial prostate-specific antigen measurements in men with and without prostate disease. *Cancer Res* **52,** 3323-8.

Casenghi, M., Meraldi, P., Weinhart, U., Duncan, P. I., Korner, R. &Nigg, E. A. (2003). Polo-like kinase 1 regulates Nlp, a centrosome protein involved in microtubule nucleation. *Dev Cell* **5,** 113-25.

Chang, S., Kim, S. H., Lim, H. K., Lee, W. J., Choi, D. &Lim, J. H. (2005). Needle tract implantation after sonographically guided percutaneous biopsy of hepatocellular carcinoma: evaluation of doubling time, frequency, and features on CT. *AJR Am J Roentgenol* **185,** 400-5.

Char, D. H., Kroll, S. &Phillips, T. L. (1997). Uveal melanoma. Growth rate and prognosis. *Arch Ophthalmol* **115,** 1014-8.

Cheeseman, I. M., Anderson, S., Jwa, M., Green, E. M., Kang, J., Yates, J. R., 3rd, *et al.* (2002). Phospho-regulation of kinetochore-microtubule attachments by the Aurora kinase Ipl1p. *Cell* **111,** 163-72.

Chen Hairong, L. A., Li Aiping, Zhou Jianwei (2004). JWA protein binds to alpha-tubulin in PC12 cells. *Chinese Science Bulletin* **49,** 467-471.

Chen, X., Kojima, S., Borisy, G. G. &Green, K. J. (2003). p120 catenin associates with kinesin and facilitates the transport of cadherin-catenin complexes to intercellular junctions. *J Cell Biol* **163,** 547-57.

Cho, Y., Gorina, S., Jeffrey, P. D. &Pavletich, N. P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science* **265,** 346-55.

Choo, R., DeBoer, G., Klotz, L., Danjoux, C., Morton, G. C., Rakovitch, E., *et al.* (2001). PSA doubling time of prostate carcinoma managed with watchful observation alone. *Int J Radiat Oncol Biol Phys* **50,** 615-20.

Chopra, P., Sethi, G., Dastidar, S. G. &Ray, A. Polo-like kinase inhibitors: an emerging opportunity for cancer therapeutics. *Expert Opin Investig Drugs* **19,** 27-43.

Colombo, R.Moll, J. (2008). Target validation and biomarker identification in oncology : the example of aurora kinases. *Mol Diagn Ther* **12,** 71-6.

Conant, E. F., Dillon, R. L., Palazzo, J., Ehrlich, S. M. &Feig, S. A. (1994). Imaging findings in mucin-containing carcinomas of the breast: correlation with pathologic features. *AJR Am J Roentgenol* **163,** 821-4.

Crosio, C., Fimia, G. M., Loury, R., Kimura, M., Okano, Y., Zhou, H., *et al.* (2002). Mitotic phosphorylation of histone H3: spatio-temporal regulation by mammalian Aurora kinases. *Mol Cell Biol* **22,** 874-85.

D. C. Smith, C. B., E. B. Garon, G. Vlahovic, R. Chugh, M. A. Lee, B. Cancilla, D. O. Clary, S. C. Robertson, H. (2010). ASCO Annual Meeting.

Dancey, J. T., Deubelbeiss, K. A., Harker, L. A. &Finch, C. A. (1976). Neutrophil kinetics in man. *J Clin Invest* **58,** 705-15.

Dark, G. G., Hill, S. A., Prise, V. E., Tozer, G. M., Pettit, G. R. &Chaplin, D. J. (1997). Combretastatin A-4, an agent that displays potent and selective toxicity toward tumor vasculature. *Cancer Res* **57,** 1829-34.

DeAngelis, L. M., Gnecco, C., Taylor, L. &Warrell, R. P., Jr. (1991). Evolution of neuropathy and myopathy during intensive vincristine/corticosteroid chemotherapy for non-Hodgkin's lymphoma. *Cancer* **67,** 2241-6.

DeLeo, A. B., Jay, G., Appella, E., Dubois, G. C., Law, L. W. &Old, L. J. (1979). Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proc Natl Acad Sci U S A* **76,** 2420-4.

Demir, O., Korulu, S., Yildiz, A., Karabay, A. &Kurnaz, I. A. (2009). Elk-1 interacts with neuronal microtubules and relocalizes to the nucleus upon phosphorylation. *Mol Cell Neurosci* **40,** 111-9.

Denduluri, N., Low, J. A., Lee, J. J., Berman, A. W., Walshe, J. M., Vatas, U., *et al.* (2007). Phase II trial of ixabepilone, an epothilone B analog, in patients with metastatic breast cancer previously untreated with taxanes. *J Clin Oncol* **25,** 3421-7.

Dingwall, C., Dilworth, S. M., Black, S. J., Kearsey, S. E., Cox, L. S. &Laskey, R. A. (1987). Nucleoplasmin cDNA sequence reveals polyglutamic acid tracts and a

cluster of sequences homologous to putative nuclear localization signals. *EMBO J* **6,** 69-74.

Dong, C., Li, Z., Alvarez, R., Jr., Feng, X. H. &Goldschmidt-Clermont, P. J. (2000). Microtubule binding to Smads may regulate TGF beta activity. *Mol Cell* **5,** 27-34.

Dufner, A., Pownall, S. &Mak, T. W. (2006). Caspase recruitment domain protein 6 is a microtubule-interacting protein that positively modulates NF-kappaB activation. *Proc Natl Acad Sci U S A* **103,** 988-93.

Dutertre, S., Cazales, M., Quaranta, M., Froment, C., Trabut, V., Dozier, C., *et al.* (2004). Phosphorylation of CDC25B by Aurora-A at the centrosome contributes to the G2-M transition. *J Cell Sci* **117,** 2523-31.

Dutertre, S., Descamps, S. &Prigent, C. (2002). On the role of aurora-A in centrosome function. *Oncogene* **21,** 6175-83.

Dvorak, Z., Vrzal, R., Ulrichova, J., Macejova, D., Ondkova, S. &Brtko, J. (2007). Expression, protein stability and transcriptional activity of retinoic acid receptors are affected by microtubules interfering agents and all-trans-retinoic acid in primary rat hepatocytes. *Mol Cell Endocrinol* **267,** 89-96.

Ebara, M., Hatano, R., Fukuda, H., Yoshikawa, M., Sugiura, N. &Saisho, H. (1998). Natural course of small hepatocellular carcinoma with underlying cirrhosis. A study of 30 patients. *Hepatogastroenterology* **45 Suppl 3,** 1214-20.

Eckerdt, F.Strebhardt, K. (2006). Polo-like kinase 1: target and regulator of anaphase-promoting complex/cyclosome-dependent proteolysis. *Cancer Res* **66,** 6895-8.

Egawa, S., Arai, Y., Tobisu, K., Kuwao, S., Kamoto, T., Kakehi, Y., *et al.* (2000). Use of pretreatment prostate-specific antigen doubling time to predict outcome after radical prostatectomy. *Prostate Cancer Prostatic Dis* **3,** 269-274.

Egawa, S., Matsumoto, K., Suyama, K., Iwamura, M., Kuwao, S. &Baba, S. (1999). Observations of prostate specific antigen doubling time in Japanese patients with nonmetastatic prostate carcinoma. *Cancer* **86,** 463-9.

El Sharouni, S. Y., Kal, H. B. &Battermann, J. J. (2003). Accelerated regrowth of non-small-cell lung tumours after induction chemotherapy. *Br J Cancer* **89,** 2184-9.

Eskelin, S., Pyrhonen, S., Summanen, P., Hahka-Kemppinen, M. &Kivela, T. (2000). Tumor doubling times in metastatic malignant melanoma of the uvea: tumor progression before and after treatment. *Ophthalmology* **107,** 1443-9.

Etienne-Manneville, S. From signaling pathways to microtubule dynamics: the key players. *Curr Opin Cell Biol* **22,** 104-11.

Filderman, A. E., Shaw, C. &Matthay, R. A. (1986). Lung cancer. Part I: Etiology, pathology, natural history, manifestations, and diagnostic techniques. *Invest Radiol* **21,** 80-90.

Finlay, I. G., Meek, D., Brunton, F. &McArdle, C. S. (1988). Growth rate of hepatic metastases in colorectal carcinoma. *Br J Surg* **75,** 641-4.

Fletcher, L. M., Welsh, G. I., Oatey, P. B. &Tavare, J. M. (2000). Role for the microtubule cytoskeleton in GLUT4 vesicle trafficking and in the regulation of insulin-stimulated glucose uptake. *Biochem J* **352 Pt 2,** 267-76.

Foord, O. S., Bhattacharya, P., Reich, Z. &Rotter, V. (1991). A DNA binding domain is contained in the C-terminus of wild type p53 protein. *Nucleic Acids Res* **19,** 5191-8.

Forsyth, P. A., Balmaceda, C., Peterson, K., Seidman, A. D., Brasher, P. &DeAngelis, L. M. (1997). Prospective study of paclitaxel-induced peripheral neuropathy with quantitative sensory testing. *J Neurooncol* **35,** 47-53.

Fujimura S, S. S., Yamauchi A, Sato H, Sohara Y, Kondo T, et al. (1979). Tumor doubling time and PPD skin test reactivity in resectable lung cancer. *J Jpn Lung Cancer Soc***,** 135-42.

Gadea, B. B.Ruderman, J. V. (2006). Aurora B is required for mitotic chromatin-induced phosphorylation of Op18/Stathmin. *Proc Natl Acad Sci U S A* **103,** 4493-8.

Galigniana, M. D., Morishima, Y., Gallay, P. A. &Pratt, W. B. (2004). Cyclophilin-A is bound through its peptidylprolyl isomerase domain to the cytoplasmic dynein motor protein complex. *J Biol Chem* **279,** 55754-9.

Ganansia-Leymarie, V., Bischoff, P., Bergerat, J. P. &Holl, V. (2003). Signal transduction pathways of taxanes-induced apoptosis. *Curr Med Chem Anticancer Agents* **3,** 291-306.

Garcia-Rocha, M., Avila, J. &Lozano, J. (1997). The zeta isozyme of protein kinase C binds to tubulin through the pseudosubstrate domain. *Exp Cell Res* **230,** 1-8.

Geddes, D. M. (1979). The natural history of lung cancer: a review based on rates of tumour growth. *Br J Dis Chest* **73,** 1-17.

Gerber, G. S., Gornik, H. L., Goldfischer, E. R., Chodak, G. W. &Rukstalis, D. B. (1998). Evaluation of changes in prostate specific antigen in clinically localized prostate cancer managed without initial therapy. *J Urol* **159,** 1243-6.

Giannakakou, P., Sackett, D. L., Ward, Y., Webster, K. R., Blagosklonny, M. V. &Fojo, T. (2000). p53 is associated with cellular microtubules and is transported to the nucleus by dynein. *Nat Cell Biol* **2,** 709-17.

Giet, R., Petretti, C. &Prigent, C. (2005). Aurora kinases, aneuploidy and cancer, a coincidence or a real link? *Trends Cell Biol* **15,** 241-50.

Giet, R., Uzbekov, R., Cubizolles, F., Le Guellec, K. &Prigent, C. (1999). The Xenopus laevis aurora-related protein kinase pEg2 associates with and phosphorylates the kinesin-related protein XlEg5. *J Biol Chem* **274,** 15005-13.

Giles, F. J., Cortes, J., Jones, D., Bergstrom, D., Kantarjian, H. &Freedman, S. J. (2007). MK-0457, a novel kinase inhibitor, is active in patients with chronic myeloid leukemia or acute lymphocytic leukemia with the T315I BCR-ABL mutation. *Blood* **109,** 500-2.

Gorlich, D., Vogel, F., Mills, A. D., Hartmann, E. &Laskey, R. A. (1995). Distinct functions for the two importin subunits in nuclear protein import. *Nature* **377,** 246-8.

Goto, H., Yasui, Y., Kawajiri, A., Nigg, E. A., Terada, Y., Tatsuka, M., *et al.* (2003). Aurora-B regulates the cleavage furrow-specific vimentin phosphorylation in the cytokinetic process. *J Biol Chem* **278,** 8526-30.

Goto, H., Yasui, Y., Nigg, E. A. &Inagaki, M. (2002). Aurora-B phosphorylates Histone H3 at serine28 with regard to the mitotic chromosome condensation. *Genes Cells* **7,** 11-7.

Graeber, T. G., Peterson, J. F., Tsai, M., Monica, K., Fornace, A. J., Jr. &Giaccia, A. J. (1994). Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. *Mol Cell Biol* **14,** 6264-77.

Gross, S. P. (2004). Hither and yon: a review of bi-directional microtubule-based transport. *Phys Biol* **1,** R1-11.

Guilherme, A., Emoto, M., Buxton, J. M., Bose, S., Sabini, R., Theurkauf, W. E., *et al.* (2000). Perinuclear localization and insulin responsiveness of GLUT4 requires cytoskeletal integrity in 3T3-L1 adipocytes. *J Biol Chem* **275,** 38151-9.

Hainaut, P.Hollstein, M. (2000). p53 and human cancer: the first ten thousand mutations. *Adv Cancer Res* **77,** 81-137.

Hanks, G. E., Hanlon, A. L., Lee, W. R., Slivjak, A. &Schultheiss, T. E. (1996). Pretreatment prostate-specific antigen doubling times: clinical utility of this predictor of prostate cancer behavior. *Int J Radiat Oncol Biol Phys* **34,** 549-53.

Harrell, J. M., Murphy, P. J., Morishima, Y., Chen, H., Mansfield, J. F., Galigniana, M. D., *et al.* (2004). Evidence for glucocorticoid receptor transport on microtubules by dynein. *J Biol Chem* **279,** 54647-54.

Harrison, M. R., Holen, K. D. &Liu, G. (2009). Beyond taxanes: a review of novel agents that target mitotic tubulin and microtubules, kinases, and kinesins. *Clin Adv Hematol Oncol* **7,** 54-64.

Herbst, R. S.Khuri, F. R. (2003). Mode of action of docetaxel - a basis for combination with novel anticancer agents. *Cancer Treat Rev* **29,** 407-15.

Heuser, L., Spratt, J. S. &Polk, H. C., Jr. (1979). Growth rates of primary breast cancers. *Cancer* **43,** 1888-94.

Horton, J. K., Houghton, P. J. &Houghton, J. A. (1988). Relationships between tumor responsiveness, vincristine pharmacokinetics and arrest of mitosis in human tumor xenografts. *Biochem Pharmacol* **37,** 3995-4000.

Hsu, L. C.White, R. L. (1998). BRCA1 is associated with the centrosome during mitosis. *Proc Natl Acad Sci U S A* **95,** 12983-8.

Huszar, D., Theoclitou, M. E., Skolnik, J. &Herbst, R. (2009). Kinesin motor proteins as targets for cancer therapy. *Cancer Metastasis Rev* **28,** 197-208.

Imamura, J., Bartram, C. R., Berthold, F., Harms, D., Nakamura, H. &Koeffler, H. P. (1993). Mutation of the p53 gene in neuroblastoma and its relationship with N-myc amplification. *Cancer Res* **53,** 4053-8.

Ito, K., Hibi, K., Ando, H., Hidemura, K., Yamazaki, T., Akiyama, S., *et al.* (2002). Usefulness of analytical CEA doubling time and half-life time for overlooked synchronous metastases in colorectal carcinoma. *Jpn J Clin Oncol* **32,** 54-8.

Jackson, J. R., Patrick, D. R., Dar, M. M. &Huang, P. S. (2007). Targeted anti-mitotic therapies: can we improve on tubulin agents? *Nat Rev Cancer* **7,** 107-17.

Jang, H. J., Hwang, S., Cho, K. Y., Kim do, K., Chay, K. O. &Kim, J. K. (2008). Taxol induces oxidative neuronal cell death by enhancing the activity of NADPH oxidase in mouse cortical cultures. *Neurosci Lett* **443,** 17-22.

Jimbo, T., Kawasaki, Y., Koyama, R., Sato, R., Takada, S., Haraguchi, K., *et al.* (2002). Identification of a link between the tumour suppressor APC and the kinesin superfamily. *Nat Cell Biol* **4,** 323-7.

Kanthou, C.Tozer, G. M. (2007). Tumour targeting by microtubule-depolymerizing vascular disrupting agents. *Expert Opin Ther Targets* **11,** 1443-57.

Kawa, G., Hiura, Y., Satoh, H., Sugi, M., Fujita, I., Oguchi, N., *et al.* (2002). [A clinical analysis of patients with early-stage prostate cancer managed without initial treatment]. *Hinyokika Kiyo* **48,** 133-8.

Kawajiri, A., Yasui, Y., Goto, H., Tatsuka, M., Takahashi, M., Nagata, K., *et al.* (2003). Functional significance of the specific sites phosphorylated in desmin at cleavage furrow: Aurora-B may phosphorylate and regulate type III intermediate filaments during cytokinesis coordinatedly with Rho-kinase. *Mol Biol Cell* **14,** 1489-500.

Kim, I. S., Kim, D. H., Han, S. M., Chin, M. U., Nam, H. J., Cho, H. P., *et al.* (2000). Truncated form of importin alpha identified in breast cancer cell inhibits nuclear import of p53. *J Biol Chem* **275,** 23139-45.

Kinoshita, K., Noetzel, T. L., Pelletier, L., Mechtler, K., Drechsel, D. N., Schwager, A., *et al.* (2005). Aurora A phosphorylation of TACC3/maskin is required for centrosome-dependent microtubule assembly in mitosis. *J Cell Biol* **170,** 1047-55.

Kishi, K., van Vugt, M. A., Okamoto, K., Hayashi, Y. &Yaffe, M. B. (2009). Functional dynamics of Polo-like kinase 1 at the centrosome. *Mol Cell Biol* **29,** 3134-50.

Koscielny, S., Tubiana, M. &Valleron, A. J. (1985). A simulation model of the natural history of human breast cancer. *Br J Cancer* **52,** 515-24.

Kubota, K., Ina, H., Okada, Y. &Irie, T. (2003). Growth rate of primary single hepatocellular carcinoma: determining optimal screening interval with contrast enhanced computed tomography. *Dig Dis Sci* **48,** 581-6.

Kunitoku, N., Sasayama, T., Marumoto, T., Zhang, D., Honda, S., Kobayashi, O., *et al.* (2003). CENP-A phosphorylation by Aurora-A in prophase is required for enrichment of Aurora-B at inner centromeres and for kinetochore function. *Dev Cell* **5,** 853-64.

Kuroishi, T., Tominaga, S., Morimoto, T., Tashiro, H., Itoh, S., Watanabe, H., *et al.* (1990). Tumor growth rate and prognosis of breast cancer mainly detected by mass screening. *Jpn J Cancer Res* **81,** 454-62.

Lam, M. H., Thomas, R. J., Loveland, K. L., Schilders, S., Gu, M., Martin, T. J., *et al.* (2002). Nuclear transport of parathyroid hormone (PTH)-related protein is dependent on microtubules. *Mol Endocrinol* **16,** 390-401.

Lane, D. P. (1992). Cancer. p53, guardian of the genome. *Nature* **358,** 15-6.

Lane, D. P.Crawford, L. V. (1979). T antigen is bound to a host protein in SV40-transformed cells. *Nature* **278,** 261-3.

Lange, A., Mills, R. E., Lange, C. J., Stewart, M., Devine, S. E. &Corbett, A. H. (2007). Classical nuclear localization signals: definition, function, and interaction with importin alpha. *J Biol Chem* **282,** 5101-5.

Lee, J. H., Gulec, S. A., Kyshtoobayeva, A., Sim, M. S. &Morton, D. L. (2009). Biological factors, tumor growth kinetics, and survival after metastasectomy for pulmonary melanoma. *Ann Surg Oncol* **16,** 2834-9.

Lee, W. R., Hanks, G. E., Corn, B. W. &Schultheiss, T. E. (1995). Observations of pretreatment prostate-specific antigen doubling time in 107 patients referred for definitive radiotherapy. *Int J Radiat Oncol Biol Phys* **31,** 21-4.

Li, M., Brooks, C. L., Wu-Baer, F., Chen, D., Baer, R. &Gu, W. (2003). Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. *Science* **302,** 1972-5.

Li, Q., Falsey, R. R., Gaitonde, S., Sotello, V., Kislin, K. &Martinez, J. D. (2007). Genetic analysis of p53 nuclear importation. *Oncogene* **26,** 7885-93.

Liang, S. H.Clarke, M. F. (1999). A bipartite nuclear localization signal is required for p53 nuclear import regulated by a carboxyl-terminal domain. *J Biol Chem* **274,** 32699-703.

Lilling, G., Nordenberg, J., Rotter, V., Goldfinger, N., Peller, S. &Sidi, Y. (2002). Altered subcellular localization of p53 in estrogen-dependent and estrogen-independent breast cancer cells. *Cancer Invest* **20,** 509-17.

Linke, S. P., Clarkin, K. C., Di Leonardo, A., Tsou, A. &Wahl, G. M. (1996). A reversible, p53-dependent G0/G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. *Genes Dev* **10,** 934-47.

Linzer, D. I.Levine, A. J. (1979). Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* **17,** 43-52.

Liu, Q., Kaneko, S., Yang, L., Feldman, R. I., Nicosia, S. V., Chen, J., *et al.* (2004). Aurora-A abrogation of p53 DNA binding and transactivation activity by phosphorylation of serine 215. *J Biol Chem* **279,** 52175-82.

Livingston, R. B., Ambus, U., George, S. L., Freireich, E. J. &Hart, J. S. (1974). In vitro determination of thymidine-3H labeling index in human solid tumors. *Cancer Res* **34,** 1376-80.

Lotti, L. V., Ottini, L., D'Amico, C., Gradini, R., Cama, A., Belleudi, F., *et al.* (2002). Subcellular localization of the BRCA1 gene product in mitotic cells. *Genes Chromosomes Cancer* **35,** 193-203.

Lowe, S. W.Ruley, H. E. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes Dev* **7,** 535-45.

Lundgren, B. (1977). Observations on growth rate of breast carcinomas and its possible implications for lead time. *Cancer* **40,** 1722-5.

Mabjeesh, N. J., Escuin, D., LaVallee, T. M., Pribluda, V. S., Swartz, G. M., Johnson, M. S., *et al.* (2003). 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. *Cancer Cell* **3,** 363-75.

Maltzman, W.Czyzyk, L. (1984). UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. *Mol Cell Biol* **4,** 1689-94.

Manavathi, B., Acconcia, F., Rayala, S. K. &Kumar, R. (2006). An inherent role of microtubule network in the action of nuclear receptor. *Proc Natl Acad Sci U S A* **103,** 15981-6.

Marchesi, V. T.Ngo, N. (1993). In vitro assembly of multiprotein complexes containing alpha, beta, and gamma tubulin, heat shock protein HSP70, and elongation factor 1 alpha. *Proc Natl Acad Sci U S A* **90,** 3028-32.

Mary, S., Charrasse, S., Meriane, M., Comunale, F., Travo, P., Blangy, A., *et al.* (2002). Biogenesis of N-cadherin-dependent cell-cell contacts in living fibroblasts is a microtubule-dependent kinesin-driven mechanism. *Mol Biol Cell* **13,** 285-301.

Matsuhashi, T., Yamada, N., Shinzawa, H. &Takahashi, T. (1996). Effect of alcohol on tumor growth of hepatocellular carcinoma with type C cirrhosis. *Intern Med* **35,** 443-8.

Matsui, T., Yao, T. &Iwashita, A. (2000). Natural history of early colorectal cancer. *World J Surg* **24,** 1022-8.

Meek, D. W. (2000). The role of p53 in the response to mitotic spindle damage. *Pathol Biol (Paris)* **48,** 246-54.

Mielke, S., Sparreboom, A. &Mross, K. (2006). Peripheral neuropathy: a persisting challenge in paclitaxel-based regimes. *Eur J Cancer* **42,** 24-30.

Milas, L., Hunter, N. R., Kurdoglu, B., Mason, K. A., Meyn, R. E., Stephens, L. C., *et al.* (1995). Kinetics of mitotic arrest and apoptosis in murine mammary and ovarian tumors treated with taxol. *Cancer Chemother Pharmacol* **35,** 297-303.

Moll, U. M., LaQuaglia, M., Benard, J. &Riou, G. (1995). Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. *Proc Natl Acad Sci U S A* **92,** 4407-11.

Munemitsu, S., Souza, B., Muller, O., Albert, I., Rubinfeld, B. &Polakis, P. (1994). The APC gene product associates with microtubules in vivo and promotes their assembly in vitro. *Cancer Res* **54,** 3676-81.

Murata-Hori, M., Fumoto, K., Fukuta, Y., Iwasaki, T., Kikuchi, A., Tatsuka, M., *et al.* (2000). Myosin II regulatory light chain as a novel substrate for AIM-1, an aurora/Ipl1p-related kinase from rat. *J Biochem* **128,** 903-7.

Nakai, R., Iida, S., Takahashi, T., Tsujita, T., Okamoto, S., Takada, C., *et al.* (2009). K858, a novel inhibitor of mitotic kinesin Eg5 and antitumor agent, induces cell death in cancer cells. *Cancer Res* **69,** 3901-9.

Nakajima, H., Toyoshima-Morimoto, F., Taniguchi, E. &Nishida, E. (2003). Identification of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate. *J Biol Chem* **278,** 25277-80.

Nakata, S., Hasumi, M., Suzuki, T., Sato, J., Imai, K., Yamanaka, H., *et al.* (1998). [PSA-doubling time immediately before the diagnosis of prostate cancer]. *Hinyokika Kiyo* **44,** 81-4.

O'Brate, A.Giannakakou, P. (2003). The importance of p53 location: nuclear or cytoplasmic zip code? *Drug Resist Updat* **6,** 313-22.

Okada, S., Okazaki, N., Nose, H., Aoki, K., Kawano, N., Yamamoto, J., *et al.* (1993). Follow-up examination schedule of postoperative HCC patients based on tumor volume doubling time. *Hepatogastroenterology* **40,** 311-5.

Okazaki, N., Yoshino, M., Yoshida, T., Suzuki, M., Moriyama, N., Takayasu, K., *et al.* (1989). Evaluation of the prognosis for small hepatocellular carcinoma based on tumor volume doubling time. A preliminary report. *Cancer* **63,** 2207-10.

Okorokov, A. L., Sherman, M. B., Plisson, C., Grinkevich, V., Sigmundsson, K., Selivanova, G., *et al.* (2006). The structure of p53 tumour suppressor protein reveals the basis for its functional plasticity. *EMBO J* **25,** 5191-200.

Oren, M.Levine, A. J. (1983). Molecular cloning of a cDNA specific for the murine p53 cellular tumor antigen. *Proc Natl Acad Sci U S A* **80,** 56-9.

Ottaggio, L., Bozzo, S., Moro, F., Sparks, A., Campomenosi, P., Miele, M., *et al.* (2000). Defective nuclear localization of p53 protein in a Chinese hamster cell line is associated with the formation of stable cytoplasmic protein multimers in cells with gene amplification. *Carcinogenesis* **21,** 1631-8.

Ouchi, M., Fujiuchi, N., Sasai, K., Katayama, H., Minamishima, Y. A., Ongusaha, P. P., *et al.* (2004). BRCA1 phosphorylation by Aurora-A in the regulation of G2 to M transition. *J Biol Chem* **279,** 19643-8.

Pasquier, E., Andre, N. &Braguer, D. (2007). Targeting microtubules to inhibit angiogenesis and disrupt tumour vasculature: implications for cancer treatment. *Curr Cancer Drug Targets* **7,** 566-81.

Pasquier, E., Carre, M., Pourroy, B., Camoin, L., Rebai, O., Briand, C., *et al.* (2004). Antiangiogenic activity of paclitaxel is associated with its cytostatic effect, mediated by the initiation but not completion of a mitochondrial apoptotic signaling pathway. *Mol Cancer Ther* **3,** 1301-10.

Peer, P. G., van Dijck, J. A., Hendriks, J. H., Holland, R. &Verbeek, A. L. (1993). Age-dependent growth rate of primary breast cancer. *Cancer* **71,** 3547-51.

Perry, J., Short, K. M., Romer, J. T., Swift, S., Cox, T. C. &Ashworth, A. (1999). FXY2/MID2, a gene related to the X-linked Opitz syndrome gene FXY/MID1, maps to Xq22 and encodes a FNIII domain-containing protein that associates with microtubules. *Genomics* **62,** 385-94.

Peukert, K., Staller, P., Schneider, A., Carmichael, G., Hanel, F. &Eilers, M. (1997). An alternative pathway for gene regulation by Myc. *EMBO J* **16,** 5672-86.

Pockwinse, S. M., Rajgopal, A., Young, D. W., Mujeeb, K. A., Nickerson, J., Javed, A., *et al.* (2006). Microtubule-dependent nuclear-cytoplasmic shuttling of Runx2. *J Cell Physiol* **206,** 354-62.

Pollack, A., Zagars, G. K. &Kavadi, V. S. (1994). Prostate specific antigen doubling time and disease relapse after radiotherapy for prostate cancer. *Cancer* **74,** 670-8.

Postma, T. J., Vermorken, J. B., Liefting, A. J., Pinedo, H. M. &Heimans, J. J. (1995). Paclitaxel-induced neuropathy. *Ann Oncol* **6,** 489-94.

Pruthi, R. S., Johnstone, I., Tu, I. P. &Stamey, T. A. (1997). Prostate-specific antigen doubling times in patients who have failed radical prostatectomy: correlation with histologic characteristics of the primary cancer. *Urology* **49,** 737-42.

Qi, M., Yu, W., Liu, S., Jia, H., Tang, L., Shen, M., *et al.* (2005). Septin1, a new interaction partner for human serine/threonine kinase aurora-B. *Biochem Biophys Res Commun* **336,** 994-1000.

Qu, L., Huang, S., Baltzis, D., Rivas-Estilla, A. M., Pluquet, O., Hatzoglou, M., *et al.* (2004). Endoplasmic reticulum stress induces p53 cytoplasmic localization and prevents p53-dependent apoptosis by a pathway involving glycogen synthase kinase-3beta. *Genes Dev* **18,** 261-77.

Quimby, B. B.Dasso, M. (2003). The small GTPase Ran: interpreting the signs. *Curr Opin Cell Biol* **15,** 338-44.

Quint, L. E., Cheng, J., Schipper, M., Chang, A. C. &Kalemkerian, G. (2008). Lung lesion doubling times: values and variability based on method of volume determination. *Clin Radiol* **63,** 41-8.

Redmond, T., Sanchez, E. R., Bresnick, E. H., Schlesinger, M. J., Toft, D. O., Pratt, W. B., *et al.* (1989). Immunofluorescence colocalization of the 90-kDa heat-shock protein and microtubules in interphase and mitotic mammalian cells. *Eur J Cell Biol* **50,** 66-75.

Reich, N. C., Oren, M. &Levine, A. J. (1983). Two distinct mechanisms regulate the levels of a cellular tumor antigen, p53. *Mol Cell Biol* **3,** 2143-50.

Ren, Y., Zhao, J. &Feng, J. (2003). Parkin binds to alpha/beta tubulin and increases their ubiquitination and degradation. *J Neurosci* **23,** 3316-24.

Rixe, O.Fojo, T. (2007). Is cell death a critical end point for anticancer therapies or is cytostasis sufficient? *Clin Cancer Res* **13,** 7280-7.

Roczniak-Ferguson, A.Reynolds, A. B. (2003). Regulation of p120-catenin nucleocytoplasmic shuttling activity. *J Cell Sci* **116,** 4201-12.

Roshak, A. K., Capper, E. A., Imburgia, C., Fornwald, J., Scott, G. &Marshall, L. A. (2000). The human polo-like kinase, PLK, regulates cdc2/cyclin B through phosphorylation and activation of the cdc25C phosphatase. *Cell Signal* **12,** 405-11.

Ross, J. L., Ali, M. Y. &Warshaw, D. M. (2008). Cargo transport: molecular motors navigate a complex cytoskeleton. *Curr Opin Cell Biol* **20,** 41-7.

Roth, D. M., Moseley, G. W., Glover, D., Pouton, C. W. &Jans, D. A. (2007). A microtubule-facilitated nuclear import pathway for cancer regulatory proteins. *Traffic* **8,** 673-86.

Sadek, A. G., Mitchell, D. G., Siegelman, E. S., Outwater, E. K., Matteucci, T. &Hann, H. W. (1995). Early hepatocellular carcinoma that develops within macroregenerative nodules: growth rate depicted at serial MR imaging. *Radiology* **195,** 753-6.

Saftoiu, A., Ciurea, T., Gorunescu, F., Rogoveanu, I. &Georgescu, C. (2004). Stochastic modeling of the tumor volume assessment and growth patterns in hepatocellular carcinoma. *Bull Cancer* **91,** E162-6.

Sahenk, Z., Barohn, R., New, P. &Mendell, J. R. (1994). Taxol neuropathy. Electrodiagnostic and sural nerve biopsy findings. *Arch Neurol* **51,** 726-9.

Sarli, V.Giannis, A. (2008). Targeting the kinesin spindle protein: basic principles and clinical implications. *Clin Cancer Res* **14,** 7583-7.

Sasai, K., Katayama, H., Stenoien, D. L., Fujii, S., Honda, R., Kimura, M., *et al.* (2004). Aurora-C kinase is a novel chromosomal passenger protein that can complement Aurora-B kinase function in mitotic cells. *Cell Motil Cytoskeleton* **59,** 249-63.

Sato, G., Saijo, Y., Uchiyama, B., Kumano, N., Sugawara, S., Fujimura, S., *et al.* (1999). Prognostic value of nucleolar protein p120 in patients with resected lung adenocarcinoma. *J Clin Oncol* **17,** 2721-7.

Schmid, H. P., Stamey, T. A., McNeal, J. E., Freiha, F. S., Redwine, E. A. &Whittemore, A. S. (1994). [Effect of the natural history on management of adenocarcinoma of the prostate]. *Urologe A* **33,** 144-8.

Schmit, T. L.Ahmad, N. (2007). Regulation of mitosis via mitotic kinases: new opportunities for cancer management. *Mol Cancer Ther* **6,** 1920-31.

Schotterl, H. D.Paul, E. (1988). [Growth dynamics of malignant melanomas. Determination of tumor doubling time based on morphometric measurements of roentgen images of lung metastases]. *Z Hautkr* **63,** 481-7.

Schweiger, S., Foerster, J., Lehmann, T., Suckow, V., Muller, Y. A., Walter, G., *et al.* (1999). The Opitz syndrome gene product, MID1, associates with microtubules. *Proc Natl Acad Sci U S A* **96,** 2794-9.

Sembritzki, O., Hagel, C., Lamszus, K., Deppert, W. &Bohn, W. (2002). Cytoplasmic localization of wild-type p53 in glioblastomas correlates with expression of vimentin and glial fibrillary acidic protein. *Neuro Oncol* **4,** 171-8.

Semiglazov, V. F., Moiseenko, V. M. &Chernomordikova, M. F. (1989). [Growth kinetics of breast cancer]. *Vopr Onkol* **35,** 288-93.

Sessa, F., Mapelli, M., Ciferri, C., Tarricone, C., Areces, L. B., Schneider, T. R., *et al.* (2005). Mechanism of Aurora B activation by INCENP and inhibition by hesperadin. *Mol Cell* **18,** 379-91.

Setou, M., Nakagawa, T., Seog, D. H. &Hirokawa, N. (2000). Kinesin superfamily motor protein KIF17 and mLin-10 in NMDA receptor-containing vesicle transport. *Science* **288,** 1796-802.

Shaulsky, G., Goldfinger, N., Ben-Ze'ev, A. &Rotter, V. (1990). Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigenesis. *Mol Cell Biol* **10,** 6565-77.

Sheu, J. C., Sung, J. L., Chen, D. S., Yang, P. M., Lai, M. Y., Lee, C. S., *et al.* (1985). Growth rate of asymptomatic hepatocellular carcinoma and its clinical implications. *Gastroenterology* **89,** 259-66.

Shieh, S. Y., Ikeda, M., Taya, Y. &Prives, C. (1997). DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* **91,** 325-34.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., *et al.* (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* **82,** 1107-12.

Smith, J. A.Martin, L. (1973). Do cells cycle? *Proc Natl Acad Sci U S A* **70,** 1263-7.

Sontag, E., Nunbhakdi-Craig, V., Bloom, G. S. &Mumby, M. C. (1995). A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. *J Cell Biol* **128,** 1131-44.

Soussi, T.Beroud, C. (2001). Assessing TP53 status in human tumours to evaluate clinical outcome. *Nat Rev Cancer* **1,** 233-40.

Spratt, J. S., Greenberg, R. A. &Heuser, L. S. (1986). Geometry, growth rates, and duration of cancer and carcinoma in situ of the breast before detection by screening. *Cancer Res* **46,** 970-4.

Spratt, J. S., Jr., Kaltenbach, M. L. &Spratt, J. A. (1977). Cytokinetic definition of acute and chronic breast cancer. *Cancer Res* **37,** 226-30.

Stamey, T. A.Kabalin, J. N. (1989). Prostate specific antigen in the diagnosis and treatment of adenocarcinoma of the prostate. I. Untreated patients. *J Urol* **141,** 1070-5.

Steegmaier, M., Hoffmann, M., Baum, A., Lenart, P., Petronczki, M., Krssak, M., *et al.* (2007). BI 2536, a potent and selective inhibitor of polo-like kinase 1, inhibits tumor growth in vivo. *Curr Biol* **17,** 316-22.

Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J. &Wahl, G. M. (1999). A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. *EMBO J* **18,** 1660-72.

Sumara, I., Vorlaufer, E., Stukenberg, P. T., Kelm, O., Redemann, N., Nigg, E. A., *et al.* (2002). The dissociation of cohesin from chromosomes in prophase is regulated by Polo-like kinase. *Mol Cell* **9,** 515-25.

Sun, X. F., Carstensen, J. M., Zhang, H., Stal, O., Wingren, S., Hatschek, T., *et al.* (1992). Prognostic significance of cytoplasmic p53 oncoprotein in colorectal adenocarcinoma. *Lancet* **340,** 1369-73.

Tada, M., Misaki, F. &Kawai, K. (1984). Growth rates of colorectal carcinoma and adenoma by roentgenologic follow-up observations. *Gastroenterol Jpn* **19,** 550-5.

Takahashi, Y., Kusama, S., Mai, M., Ueno, M., Ogino, T., Ueda, H., *et al.* (1986). [Study of CEA doubling time in liver metastasis from colonic cancer and postoperative survival time]. *Nippon Geka Gakkai Zasshi* **87,** 1422-5.

Takesue, F., Inutsuka, S., Nagahama, S., Kusumoto, H., Korenaga, D., Maekawa, S., *et al.* (1998). [Serum carcinoembryonic antigen doubling time in patients with recurrent gastrointestinal carcinoma and its relationship to tumor biology and life expectancy]. *Gan To Kagaku Ryoho* **25 Suppl 3,** 464-8.

Taouli, B., Goh, J. S., Lu, Y., Qayyum, A., Yeh, B. M., Merriman, R. B., *et al.* (2005). Growth rate of hepatocellular carcinoma: evaluation with serial computed tomography or magnetic resonance imaging. *J Comput Assist Tomogr* **29,** 425-9.

Taylor, S.Peters, J. M. (2008). Polo and Aurora kinases: lessons derived from chemical biology. *Curr Opin Cell Biol* **20,** 77-84.

Teckchandani, A. M., Birukova, A. A., Tar, K., Verin, A. D. &Tsygankov, A. Y. (2005). The multidomain protooncogenic protein c-Cbl binds to tubulin and stabilizes microtubules. *Exp Cell Res* **306,** 114-27.

Teicher, B. A. (1997). *Anticancer Drug Development Guide; Preclinical Screening, Clinical Trials, and Approval*. Humana Press.

Tezuka, M., Hayashi, K., Kubota, K., Sekine, S., Okada, Y., Ina, H., *et al.* (2007). Growth rate of locally recurrent hepatocellular carcinoma after transcatheter arterial chemoembolization: comparing the growth rate of locally recurrent tumor with that of primary hepatocellular carcinoma. *Dig Dis Sci* **52,** 783-8.

Tilanus-Linthorst, M. M., Kriege, M., Boetes, C., Hop, W. C., Obdeijn, I. M., Oosterwijk, J. C., *et al.* (2005). Hereditary breast cancer growth rates and its impact on screening policy. *Eur J Cancer* **41,** 1610-7.

Toji, S., Yabuta, N., Hosomi, T., Nishihara, S., Kobayashi, T., Suzuki, S., *et al.* (2004). The centrosomal protein Lats2 is a phosphorylation target of Aurora-A kinase. *Genes Cells* **9,** 383-97.

Toyoshima-Morimoto, F., Taniguchi, E., Shinya, N., Iwamatsu, A. &Nishida, E. (2001). Polo-like kinase 1 phosphorylates cyclin B1 and targets it to the nucleus during prophase. *Nature* **410,** 215-20.

Tran, T. A., Gillet, L., Roger, S., Besson, P., White, E. &Le Guennec, J. Y. (2009). Non-anti-mitotic concentrations of taxol reduce breast cancer cell invasiveness. *Biochem Biophys Res Commun* **379,** 304-8.

Trostel, S. Y., Sackett, D. L. &Fojo, T. (2006). Oligomerization of p53 precedes its association with dynein and nuclear accumulation. *Cell Cycle* **5,** 2253-9.

Tsutsumi, T., Kosaka, T., Ushiro, H., Kimura, K., Honda, T., Kayahara, T., *et al.* (2008). PASK (proline-alanine-rich Ste20-related kinase) binds to tubulin and microtubules and is involved in microtubule stabilization. *Arch Biochem Biophys* **477,** 267-78.

Tubiana M, M. E. (1976). Growth rate and cell kinetics in human tumours: some prognostic and therapeutic implications. *In: Symington T, Carter RL, eds. Scientific Foundations of Oncology. Chicago:Year Book Medical Publishers***,** 126-36.

Umehara, Y., Kimura, T., Yoshida, M., Oba, N. &Harada, Y. (1993). Comparison of doubling times of serum carcinoembryonic antigen produced by various metastatic lesions in recurrent gastric and colorectal carcinomas. *Cancer* **71,** 4055-9.

Umetani, N., Masaki, T., Watanabe, T., Sasaki, S., Matsuda, K. &Muto, T. (2000). Retrospective radiographic analysis of nonpedunculated colorectal carcinomas with special reference to tumor doubling time and morphological change. *Am J Gastroenterol* **95,** 1794-9.

Usuda, K., Saito, Y., Sagawa, M., Sato, M., Kanma, K., Takahashi, S., *et al.* (1994). Tumor doubling time and prognostic assessment of patients with primary lung cancer. *Cancer* **74,** 2239-44.

Utama, B., Shen, Y. H., Mitchell, B. M., Makagiansar, I. T., Gan, Y., Muthuswamy, R., *et al.* (2006). Mechanisms for human cytomegalovirus-induced cytoplasmic p53 sequestration in endothelial cells. *J Cell Sci* **119,** 2457-67.

van de Weerdt, B. C.Medema, R. H. (2006). Polo-like kinases: a team in control of the division. *Cell Cycle* **5,** 853-64.

van Rossum, D., Kuhse, J. &Betz, H. (1999). Dynamic interaction between soluble tubulin and C-terminal domains of N-methyl-D-aspartate receptor subunits. *J Neurochem* **72,** 962-73.

Venoux, M., Basbous, J., Berthenet, C., Prigent, C., Fernandez, A., Lamb, N. J., *et al.* (2008). ASAP is a novel substrate of the oncogenic mitotic kinase Aurora-A: phosphorylation on Ser625 is essential to spindle formation and mitosis. *Hum Mol Genet* **17,** 215-24.

von Fournier, D., Weber, E., Hoeffken, W., Bauer, M., Kubli, F. &Barth, V. (1980). Growth rate of 147 mammary carcinomas. *Cancer* **45,** 2198-2207.

Vrzal, R., Daujat-Chavanieu, M., Pascussi, J. M., Ulrichova, J., Maurel, P. &Dvorak, Z. (2008). Microtubules-interfering agents restrict aryl hydrocarbon receptor-mediated CYP1A2 induction in primary cultures of human hepatocytes via c-jun-N-terminal kinase and glucocorticoid receptor. *Eur J Pharmacol* **581,** 244-54.

Walker, K. K.Levine, A. J. (1996). Identification of a novel p53 functional domain that is necessary for efficient growth suppression. *Proc Natl Acad Sci U S A* **93,** 15335-40.

Wang, J., Lu, J. P., Tian, J. M., Wang, F., Liu, Q., Wang, L., *et al.* (2005). [Natural history of small hepatocellular carcinoma: studied by MRI]. *Zhonghua Zhong Liu Za Zhi* **27,** 222-5.

Wang, X., Michael, D., de Murcia, G. &Oren, M. (2002). p53 Activation by nitric oxide involves down-regulation of Mdm2. *J Biol Chem* **277,** 15697-702.

Weinstein, J. N., Myers, T. G., O'Connor, P. M., Friend, S. H., Fornace, A. J., Jr., Kohn, K. W., *et al.* (1997). An information-intensive approach to the molecular pharmacology of cancer. *Science* **275,** 343-9.

Weiss, J., Heine, M., Korner, B., Pilch, H. &Jung, E. G. (1995). Expression of p53 protein in malignant melanoma: clinicopathological and prognostic implications. *Br J Dermatol* **133,** 23-31.

Wheatley, S. P., Carvalho, A., Vagnarelli, P. &Earnshaw, W. C. (2001). INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis. *Curr Biol* **11,** 886-90.

Winer-Muram, H. T., Jennings, S. G., Tarver, R. D., Aisen, A. M., Tann, M., Conces, D. J., *et al.* (2002). Volumetric growth rate of stage I lung cancer prior to treatment: serial CT scanning. *Radiology* **223,** 798-805.

Wolberg, W. H.Ansfield, F. J. (1971). The relation of thymidine labeling index in human tumors in vitro to the effectiveness of 5-fluorouracil chemotherapy. *Cancer Res* **31,** 448-50.

Wolf, D., Admon, S., Oren, M. &Rotter, V. (1984). Abelson murine leukemia virus-transformed cells that lack p53 protein synthesis express aberrant p53 mRNA species. *Mol Cell Biol* **4,** 552-8.

Wu, B., Decourt, B., Zabidi, M. A., Wuethrich, L. T., Kim, W. H., Zhou, Z., *et al.* (2008). Microtubule-mediated Src tyrosine kinase trafficking in neuronal growth cones. *Mol Biol Cell* **19,** 4611-27.

Xu, Y. (2003). Regulation of p53 responses by post-translational modifications. *Cell Death Differ* **10,** 400-3.

Yamamoto, M., Maehara, Y., Sakaguchi, Y., Mine, H., Yamanaka, T., Korenaga, D., *et al.* (2004). Distributions in CEA doubling time differ in patients with recurrent colorectal carcinomas. *Hepatogastroenterology* **51,** 147-51.

Yoshino, M. (1983). Growth kinetics of hepatocellular carcinoma. *Jpn J Clin Oncol* **13,** 45-52.

Yu, H., Diamandis, E. P., Prestigiacomo, A. F. &Stamey, T. A. (1995). Ultrasensitive assay of prostate-specific antigen used for early detection of prostate cancer relapse and estimation of tumor-doubling time after radical prostatectomy. *Clin Chem* **41,** 430-4.

Zeitlin, S. G., Shelby, R. D. &Sullivan, K. F. (2001). CENP-A is phosphorylated by Aurora B kinase and plays an unexpected role in completion of cytokinesis. *J Cell Biol* **155,** 1147-57.

Zhang, H., Shi, X., Paddon, H., Hampong, M., Dai, W. &Pelech, S. (2004). B23/nucleophosmin serine 4 phosphorylation mediates mitotic functions of polo-like kinase 1. *J Biol Chem* **279,** 35726-34.

Zhang, Y.Xu, W. (2008). Progress on kinesin spindle protein inhibitors as anti-cancer agents. *Anticancer Agents Med Chem* **8,** 698-704.

Zhu, M. L., Horbinski, C. M., Garzotto, M., Qian, D. Z., Beer, T. M. &Kyprianou, N. Tubulin-targeting chemotherapy impairs androgen receptor activity in prostate cancer. *Cancer Res* **70,** 7992-8002.

Ziegelbauer, J., Shan, B., Yager, D., Larabell, C., Hoffmann, B. &Tjian, R. (2001). Transcription factor MIZ-1 is regulated via microtubule association. *Mol Cell* **8,** 339-49.

11. Publications

This thesis is based on the following publications:

1. Komlodi-Pasztor E, Sackett D, Wilkerson J, Fojo T. Mitosis is not a key target of microtubule agents in patient tumors. Nature Reviews Clinical Oncology. Nat Rev Clin Oncol. 2011 Feb 1;8(4):244-50.

2. Komlodi-Pasztor E, Trostel S, Sackett D, Poruchynsky M, Fojo T. Impaired p53 binding to importin: a novel mechanism of cytoplasmic sequestration identified in oxaliplatin-resistant cells. Oncogene. 2009 Sep 3;28(35):3111-20. Epub 2009 Jul 6.

Other publications:

1. Kemecsei P, Miklós Z, Bíró T, Marincsák R, Tóth BI, Komlódi-Pásztor E, Barnucz E, Mirk E, Van der Vusse GJ, Ligeti L, Ivanics T. Hearts of surviving MLP-KO mice show transient changes of intracellular calcium handling. Mol Cell Biochem. 2010 Sep;342(1-2):251-60

2. Zsáry A, Szücs S, Keltai K, Pásztor E, Schneider T, Rosta A, Sármán P, Jánoskuti L, Fenyvesi T, Karádi I. Endothelin-1 and cardiac function in anthracycline-treated patients: a 1-year follow-up. J Cardiovasc Pharmacol. 2004 Nov;44 Suppl 1:S372-5

3. Zsáry A, Pásztor E, Szücs Sz, Keltai K, Schneider T, Rosta A, Sármán P, Jánoskuti L, Fenyvesi T, Karádi I. Endothelin-1 szintjének változása és anthracyclin okozta cardiomyopathia. Magyar Belorvosi Archivum 2004;57(2):67-70

12. Acknowledgement

I would like to express my gratitude to Dr. Tito Fojo who gave me the opportunity to work in his laboratory and who has been teaching me for years. Working with him has shaped the way I think about science and opened my eyes to the important and challenging role that scientist-physicians play in today' s scientific world.

I would like to say many thanks for Dr. Dan Sackett for helping and teaching me. His contribution to both of the articles included in my thesis was very valuable and is highly appreciated.

I am thankful to Dr. Andras Varadi for his help and support.

I am very thankful for the help and guidance of Dr. Susan Bates.

I am grateful for the help I received from members of the laboratory of Dr. Fojo and Dr. Bates, namely Shana Trostel, Lyn Huff, Dr. Marianne Poruchynsky, Julia Wilkerson, Rob Robey, and Victoria Luchenko among others. I am also very thankful for the productive collaboration with Robert Murphy.

I am also thankful for the support of my family and friends.

I am thankful for the administrative help of Dr. Ágota Vér, the Semmelweis University PhD School Doctoral Secretariat, the secretary of Professor Mandl, and the support of the Office of Intramural Training & Education, NIH.