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.

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Introduction

The level and localization of p53, often referred to as 'the guardian of the genome', is tightly regulated. Normally, p53 shuttles between the cytoplasm and the nucleus. In the cytoplasm, p53 oligomerization is followed by its association with dynein, the minus-end directed motor protein that travels on microtubules. As dynein cargo, p53 reaches the perinuclear region where it associates with importin, through one or possibly more than one nuclear localization signal (NLSI, NLSII, NLSIII) and translocates to the nucleus. Previous studies have concluded that NLSI is most important in the interaction with importin, assigning supportive roles to NLSII and NLSIII. In the nucleus, p53 initiates sequence-specific gene transcription of numerous genes involved in cell cycle arrest or apoptosis, and of Mdm2, its negative regulator. p53 ubiquitination by Mdm2 induces a conformational change that allows exportin 1 to bind to the nuclear export signal (NES) of p53. Exportin 1 translocates p53 to the cytoplasm where it undergoes proteasomal degradation.

Long-distance intracellular trafficking of p53 (and other proteins) occurs on microtubules. The disruption of the microtubule network by microtubule targeting agents, such as taxol or vincristine, leads to p53 cytoplasmic accumulation. Microtubule targeting agents are widely used as a first line therapy in the treatment of a variety of human cancers such as breast cancer, colorectal cancer, ovarian cancer, prostate cancer and lymphomas. 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 led 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. Following responses seen in preclinical models, clinical trails did not deliver on this expectation. Based on these results we now have data that validate a paradigm for how MTAs kill cancer cells in patients—principally by interfering with interphase microtubule functions, for example intracellular trafficking—not only by disrupting mitosis.

Purpose of the study

TP53 is one of the most frequently mutated genes of 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 our study, KB-3.1 cells, a subclone of KB cells, were selected with either cisplatin or oxaliplatin. 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 a deletion of a single nucleotide in the sequence encoding for amino acid 382 in the putative nuclear localization signal III (NLS III) region in the oxaliplatin selected cells. 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 intracellular trafficking is summarized in the second half of 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.

Methods

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. 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% CO2.

Cytotoxicity Assay

Four-day cytotoxicity assays were performed using the sulforhodamine B based on the method of Skehan et al (Skehan et al., 1990). Each concentration was tested in triplicate.

Western Blot

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. 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.

Subcellular Fractionation
Cells incubated overnight were harvested and fractionated using NE-PER nuclear and cytoplasmic extraction reagent according to the manufacturer's instruction. Densitomery values were obtained using the Odyssey Li-Core program.

Immunohistochemistry
Exponentially growing cells were plated on 12-mm glass coverslips and incubated overnight. The following day cells were rinsed and fixed with 3.7% formaldehyde/PBS. Cells were then fixed in 90% methanol and coverslips were rinsed with PBS and incubated in blocking solution. Primary and secondary antibodies were added sequentially for 1 h each at room temperature.

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.

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), homogenized, and centrifuged. Lysates were pre-cleared then incubated with IC74 anti-dynein or anti-importin ß. Protein and antibody were then incubated with ImmunoPure Plus Immobilized Protein A for 2 hours at 4˚. Samples were washed 5x with 500 ml of IP buffer. The Protein A pellet was re-suspended in 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.

Mutagenesis
Wild type and mutant p53 were amplified by polymerase chain reaction using a pcDNA3.1+ vector containing wild type p53 as a template. DNA sequencing from both directions was used to confirm the sequence of the constructs.

Transfection
PC3 cells were plated and allowed to attach overnight. The following day, the PC3 cells were transiently transfected with p53 constructs using Lipofectamine 2000 according to manufacturer's instruction. Following the 6 hour-long transfection period, sub-cellular fractionation was performed immediately or following drug treatment.

Luciferase reporter assays
PC3 cells in 12-well plates were transiently transfected using TransFast Transfection Reagent for 48h. The transfection media was replaced with RPMI 5h after transfection. Vectors used included the backbone or the p21-Luc vector, the pRL-TK internal control vector, and either the wild type p53 vector or the $p53^{420}$ vector. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System.

Results

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. We showed 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.

Mutant p53 in the resistant KB cell lines

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 $p53^{420}$. Since this deletion is present in all three oxaliplatin resistant sublines, one can assume it occurred early in the selection.

Nuclear localization of $p53^{420}$

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. 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.

Microtubular trafficking of $p53^{420}$

We began by examining the steps in the cytoplasm that lead to accumulation of p53 in the nucleus. To examine the ability of $p53^{420}$ to oligomerize, we performed experiments with the cross-linking reagent, diamide. By western blot, we demonstrated cross-linked p53 oligomers in the diamide treated oxaliplatin resistant samples. Furthermore, we showed that $p53^{420}$ immunoprecipitated 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.

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

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. Since Leptomycin B had no effect on the subcellular localization of $p53^{420}$, it indicates that the preferential cytoplasmic localization of $p53^{420}$ is not a result of augmented nucleo-cytoplasmic transport.

Neither NLSII nor NLSIII are required for p53 nuclear import

To evaluate the importance of NLS II and NLS III signals in p53 nuclear import, nine p53 constructs were generated with either one or both nuclear localization signals destroyed and of either normal length or 420 amino acids in length - the length of $p53^{420}$. Following transfection in PC3 cells, the distribution of the p53 constructs was determined by sub-cellular fractionation. 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.

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

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. The level of $p53^{420}$ remained stable without drug administration; however, it declined rapidly after cycloheximide treatment. This result showed that $p53^{420}$ is relatively instable.

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

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. The association of $p53^{420}$ with importin was impaired in the KB-OX60. We thus concluded that $p53^{420}$ is sequestered in the cytoplasm because it is excluded from entering the nucleus as a consequence of poor association with importin.

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

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, cells were co-transfected with a p21-vector and a vector containing a mutant or wild type p53. $p53^{420}$ 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.

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 has 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.

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 xenographt models. During immortalization the doubling time of primary

tumor cells significantly decreases; *in vitro*, doubling times are usually less than 24 hours. Cell lines have slightly longer doubling times as xenograft models. In a rapidly dividing cell line the consequence of adding a microtubule-targeting agent is mitotic arrest. 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 led to the search for more mitosis-specific drugs, which in turn produced the mitotic kinase inhibitors.

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. Many of these newly described proteins are only functional during mitosis. 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. *In vitro* and xenograft models show extensive mitotic arrest followed by cell death upon treatment with a mitotic kinase inhibitor. It is not surprising that cells with rapid doubling times (such as *in vitro* and xenograft models) are vulnerable to mitotic poisons. But can a group of drugs that is completely dependent on cell division be successful in treating slower growing human tumors?

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. This unbiased and extensive but not exhaustive compilation of

literature data shows that the median doubling time of all tumors is approximately 147 days. 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. Longer doubling times of human tumors do not come with extended cell cycle duration. The fraction of time spent in mitosis and in turn the fraction of cells expressing the desired "mitotic specific target" are small. It is supported by the fact that 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.

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 is equal to 6.1×10^{10} cells/day in a 70 kg subject. 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.

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. 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. 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.

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. 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.

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.

Conclusions

- 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 $p53^{420}$ 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 (MTA) impairs protein translocation to the nucleus.

- MTAs 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.

- Following promising results arising from preclinical models, mitotic kinase inhibitors (MKIs) 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 MKIs (febrile neutropenia) proves that these drugs are well-designed, target rapidly dividing cells, and have excellent bioavailability.

- The clinical failure of MKIs stands as an evidence that MTAs target cells not only in mitosis but also in interphase.

- Neurotoxicity stands as strong evidence that MTAs target non-dividing cells too.

- MTAs kill cancer cells in patients—principally by interfering with interphase microtubule functions, for example intracellular trafficking—not only by disrupting mitosis.

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. Nature Reviews Clinical Oncology. 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.

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