Nutlins: A Novel Therapeutic Strategy for Inhibiting the MDM2-p53 Interaction in Cancer?

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ABSTRACT

p53 is well established as a tumour suppressor and extensive study has been done on how p53 mediates the cell cycle and its role in apoptosis and cancer. It has also been shown that p53 is frequently found mutated in human cancers but recent evidence suggests wild-type p53 also plays a significant role. The regulation of p53 in cancers with wild-type p53 has become the focus of much study. MDM2, the biological regulator of p53, is found over-expressed in some cancers and has become the target of intensive research. The quest for inhibitors in the MDM2-p53 complex has led to the development of a group of *cis*-imidazoline analogues which have been shown to cause selective cancer cell death, *in vitro* and *in vivo*. Nutlins, a family of *cis*-imidazoline analogues, are small-molecule MDM2 antagonists, based on the structural relationship between p53 and MDM2 and have the potential for target specificity that is not seen in current anti-cancer therapies.

INTRODUCTION

It is predicted that in 2005, there will be more mortalities from cancer than from heart disease in persons younger than 85 years of age.[1] In over 50 percent of cancers, the tumour suppressor gene p53 is known to be mutated.[2] Clinical evidence of this can be seen in the Li-Fraumeni syndrome where there is a germ line mutation of p53, which greatly increases the risk of developing several types of cancer including osteosarcoma, soft tissue sarcoma, breast cancer, brain tumours, adrenocortical carcinoma and leukaemia.[3]

Cancer is the dysregulation of cell homeostasis and the governing mechanisms which control growth and replication. There are six mechanisms which contribute to the development of cancer: self-sufficiency in growth signals, sustained angiogenesis, limitless replicative potential, tissue invasion and metastasis, evasion of apoptosis and insensitivity to anti-growth signals. These six characteristics are shared by most if not all human cancers.[4] p53 plays an integral role in two of these, apoptosis and the evasion of anti-growth signals.

Since its discovery in 1979, p53 has become one of the most intensively studied proteins in oncology research. Over the last 20 years, research on p53 has provided insight into cell regulation, apoptosis and the mechanisms involved in the progression of cancer. Using x-ray crystallography and emerging computer modelling techniques, p53 has propelled cancer research and cancer drug development into what it is today.

The Cell Cycle and Regulation

When a cell is not actively undergoing or preparing for mitosis it is said to be in the quiescent state, G_0. When a cell begins the process of replication it must go through certain phases, collectively known as the cell cycle. These phases include G_1, S, G_2 and M. During the G_1 phase the cell starts to increase in size and preparation for chromosome replication follows. DNA is duplicated during the S phase. The G_2 phase takes place when the cell prepares for mitosis, which occurs in the M phase. The whole process results in two identical daughter cells[3]

Progression of the cell cycle is monitored at certain points to ensure that the environment is favourable for replication and that DNA synthesis has occurred without error. These check points dictate the progression of cell replication and are activated by negative intracellular signals. If something goes wrong, the regulatory mechanisms become active.[5]

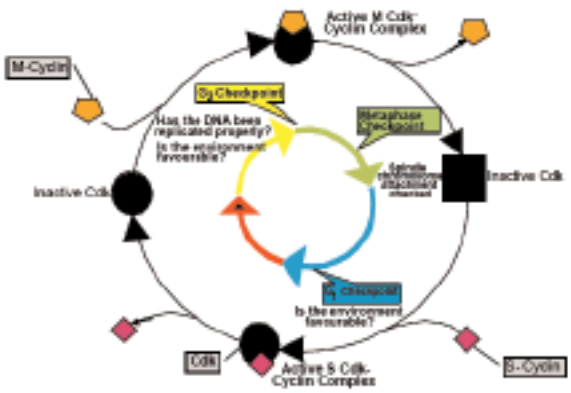

Figure 1. *The Cell Cycle.*

There are three major check points in cell cycle control (Figure 1). The most crucial to p53 are the G_1 and G_2 check points. If the DNA has been damaged, for example by exposure to radiation or by improper DNA replication, p53 will mediate through Cyclin-Cdk complexes. Inhibition of G_1/S-Cdk complex (Cyclin E, Cdk2) and the S-Cdk complex (Cyclin A, Cdk2) is achieved by p53 stimulating transcription of a cyclin kinase inhibitory (CKI) protein, p21 (Figure 2). Progression to the M phase requires Cdk1 which is also inhibited by p21 and other CKIs; in turn, CKIs are upregulated by p53 in the presence of damaged DNA.[3,6,7]

In the event that the DNA is unable to be repaired, p53 stimulates a cascade of cellular events known as apoptosis, a form of programmed cell death. Under normal circumstances, when cell DNA is damaged to the point where repair is impossible, apoptosis is triggered. Several proteins are integral in the apoptotic pathway, including p53, Bcl-2, cytochrome *c* and the caspases.[8,9]

Ultimately, stimulation of the caspase signalling pathway initiates the activation of enzymes that cleave proteins essential for cell survival and lead to apoptosis.[9] Caspases are the principal effectors of apoptosis and their activation leads to characteristic morphological changes to the cell such as shrinkage, chromatin condensation, DNA fragmentation and plasma membrane blebbing.[9]

The Functional Inhibition of p53

Through the mechanisms discussed above, p53 prevents replication of cells with irreparable DNA damage. As a result, a consistent finding is that many cancers express dysfunctional or mutated p53. This type of p53 dysregulation is observed in many forms of cancer where the inhibition of p53 is due to an over-expression or amplification of the protein, MDM2.[10]

MDM2 is a biological moderator of p53 and under normal circumstances has very low intracellular concentrations. MDM2 leads to suppression of p53 by three mechanisms. Firstly, MDM2 can bind p53 at its transactivation domain, blocking its ability to activate transcription of genes encoding proteins that repair DNA or direct apoptosis. Secondly, it is involved in nuclear export of p53. Thirdly, MDM2 may act as a ubiquitin ligase that targets p53 for degradation by proteasomes.[11]

When DNA becomes damaged, p53 is indirectly activated by multiple protein kinases, such as ATM, that phosphorylate p53 at serine and threonine residues. Phosphorylation of p53 inhibits MDM2 from binding, causing dissociation of the MDM2-p53 complex and leading to increased levels of p53 in the cell. However, once the DNA damage has been repaired the protein kinases are no longer active and phosphorylation of p53 ceases. As a result, p53 is quickly dephosphorylated and the MDM2-p53 complex reformed.[12]

p53 and Drug Development

The MDM2-p53 interaction has provided scientists with a unique opportunity for drug development. Stimulating the tumour suppressor activity of wild-type p53 protein has long been shown to eradicate tumour cells in animal models, which makes the molecule an attractive therapeutic target for drug development. Inhibition of the MDM2-p53 interaction with synthetic molecules might therefore lead to the accumulation of active p53 followed by the death of the tumour cells from apoptosis.[3,11]

MDM2 contains a specific site at which p53 binds *in vivo*. The over-expression of MDM2 inhibits wild-type p53 from responding to DNA damage. However, if the site at which MDM2 binds p53 could be blocked, perhaps by binding to an analogue of p53, this may disrupt the inhibitory effects of MDM2 on p53. If such an analogue could be designed it could prove extremely useful for targeting cancer. In addition, specifically targeting the MDM2-p53 interaction may decrease the potential for systemic toxicity by focusing treatment on cancers with a high concentration of MDM2-p53 complex.

Nutlins – Small Molecule MDM2 Antagonists

In 2004, Vassilev and co-workers (Hoffman-La Roche Inc., Nutley, New Jersey) described a class of antagonists that inhibited the MDM2-p53 complex. These antagonists are a group of *cis*-imidazoline analogues designated as the Nutlins. Through x-ray crystallography, the MDM2-p53 complex showed a well defined hydrophobic cleft which represented the binding site for p53. In addition, the structure revealed that this cleft was filled by only three side chains of the helical region of p53: Phe^{19}, Leu^{26} and Trp^{23}. This revelation lead to the possibility that a small molecular inhibitor could mimic these three amino

acids and their orientation. The inhibitor could disrupt the MDM2-p53 interaction by binding specifically in this cleft, liberating functional p53.[11]

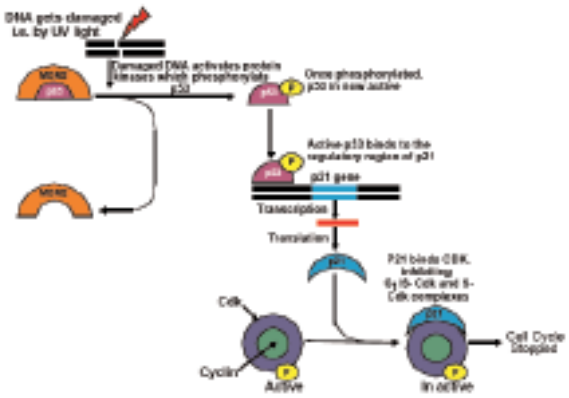

Figure 2. *Indirect inhibition of Cdk-cyclin complexes by p53.*

A combination of high-throughput screening and computer modelling, lead to the development of the Nutlins. In theory, the Nutlins should bind to the hydrophobic cleft of MDM2 displacing p53. The compounds were synthesised as a racemic mixture. However, one enantiomer exhibited significantly higher binding affinity for the p53 binding site on MDM2 compared to others. The active enantiomers of the *cis*-imidazoline analogues were arbitrarily named Nutlin-1, -2 and -3, with Nutlin-3 having the smallest inhibitory concentration (IC_{50}) values. The mode of binding of these compounds with MDM2 was determined through crystallization studies which detailed the three-dimensional structures of both molecules. It was observed that Nutlins mimicked the binding of the helical region of p53 by interacting with the hydrophobic cleft of MDM2.[11]

In order to investigate what effect these molecules would have on the activation of p53 pathways, Vassilev and colleagues studied cancer cell lines with wild-type p53. Other cell lines employing mutated p53 were used as negative controls. In the event that Nutlins did disrupt the MDM2-p53 interaction an increase in p53, p21 and MDM2 would be observed, presumably through the activation of the p53 pathway. The investigations showed that wild-type p53 cancer cells incubated with Nutlin-1 for eight hours led to a dose-dependent increase in the cellular levels of p53, MDM2 and p21. This increase was not observed in the negative controls. Similar results were seen in the other cell lines with wild-type p53 and mutant p53.[11]

Initial results indicated that MDM2 stimulated the p53 pathway in wild-type p53 cancer cell lines, but delineating the mode of activation was important. As MDM2 targets p53 for degradation, it was necessary to determine that the increase in p53 was due to decreased degradation of p53 rather than increased gene expression. Wild-type p53 cell lines were treated with Nutlins and results confirmed that p53 accumulation was due to activation of the p53 pathway via a post-translational mechanism rather than through up-regulation of p53 gene expression.[11]

Once the p53 pathway is activated, proliferating cells should arrest in the G_1 and G_2 phases of the cell cycle. At 24 hours post-treatment with Nutlins, a significant increase in G_1 and G_2/M fractions was observed with near depletion of S phase fractions, an indication that cell cycle arrest had occurred.[11] This alteration in cell cycle fractions was observed in wild-type cancer cell lines but not in mutant p53 cancer cell lines providing further evidence that Nutlins activate the p53 pathway in non-mutants.

Vassilev and co-workers also investigated the ability of the treated cells to undergo apoptosis in cancer cells with wild-type p53. An osteosarcoma cell line (SJSA-1) was treated with Nutlins for 24 and 48 hours and subsequently examined for apoptotic cells. After 48 hours of treatment 45 percent of cells were apoptotic. SJSA-1 cells were then treated again with Nutlins in the presence of a pan-caspase inhibitor for 48 hours.[11] The number of apoptotic cells decreased to 5 percent. The decrease in apoptotic cells in the presence of the pan-caspase inhibitor suggested that p53-mediated apoptosis was being activated through the caspase cascade. Therefore, the apoptotic activity of p53 had been restored post-treatment with Nutlins.

If Nutlins showed specificity for wild-type p53 cancer cells, non-cancer cells should retain viability while wild-type p53 cancer cell viability would be lost. Human and mouse fibroblast cells with a functional p53 pathway were treated with up to 10 µM of Nutlins for seven days. Nutlins inhibited the exponential proliferation of human skin cells and mouse embryo fibroblasts. After seven days the cells were still viable. SJSA-1 cells were treated concurrently and were inhibited at 1.5 µM IC_{50} but viability was lost after three days.[11] The results showed that the wild-type p53 cancer cells lost viability at lower concentrations which would imply a higher level of susceptibility and selectivity.

Finally, Vassilev and co-workers investigated the ability of Nutlins to inhibit tumour xenografts of a human osteosarcoma cell line (SJSA-1) in nude mice. Oral administration for 20 days achieved a steady-state plasma level above the IC_{90} in vitro for SJSA-1. Treatment of these mice with tumour cell xenografts resulted in 90 percent of tumour growth relative to controls. In addition, the oral administration was well tolerated and the mice did not lose any significant amount of weight nor show any gross abnormalities upon necropsy post-treatment, suggesting a favourable toxicity profile.[11] There have been no human studies with Nutlins and the potentially toxic effects in humans are still unknown. Testing to date has involved only animals where the there have been no adverse side effects documented. Since Nutlins specifically target cancer cells with an over expression of MDM2 and those which have retained wild-type p53, researchers are cautiously optimistic that treatment with Nutlins will result in minimal side effects in human subjects.

CONCLUSIONS

The past 25 years have been pivotal in furthering our understanding of cancer and its treatment. Through comprehensive research in the molecular biology of cell replication, we have come to understand much more about the cell cycle and the oncogenic factors that induce cancer. In the words of Frank McCormick, director of the University of California San Francisco Comprehensive Cancer Centre, "The black box is gone, we can see what is wrong in cancer cells, what cancer cells do to survive and how they can be vulnerable to treatment." At the centre of the black box is p53.

The intensive research on p53 has furthered the development of novel anti-cancer drugs. The identification of proteins which regulate the cell cycle has changed the approach to drug design, steering it to a more target-specific approach based on crystal structure. Nutlins have provided a drug model based on structure and protein-protein interaction, which can hopefully be applied to other such interactions and lead to further developments. At a time when cancer is replacing heart disease as the leading cause of death, it is encouraging that such strides are being made.

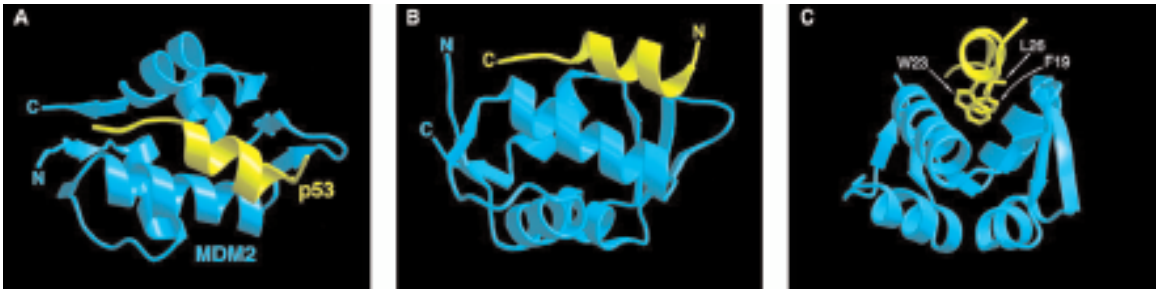

Figure 3. *P53-MDM2 interaction.*[10]

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