

Department of Microbiology, Tumor and Cell biology
Karolinska Institutet, Stockholm, Sweden

**p53 REACTIVATION BY THE SMALL
MOLECULE RITA: MOLECULAR
MECHANISMS**

Vera Grinkevich



**Karolinska
Institutet**

Stockholm 2011

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Cover Illustration. “Guardian of the genome” - puzzle.

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ISBN 978-91-7457-289-6

*To my family,
Mama, Aurel and Maria*

ABSTRACT

Inactivation of the tumor suppressor p53 is essential for the development and maintenance of cancer cells. Therefore, reactivation of p53 appears to be a promising strategy for anti-cancer therapy. We have previously identified the small molecule RITA that prevents interaction between p53 and its negative regulator Mdm2 by direct binding to p53 (Issaeva et al., 2004). RITA reactivates the transcriptional transactivation function of wild type p53 and induces p53-dependent apoptosis *in vitro* and *in vivo*.

In this thesis we addressed the molecular mechanisms of RITA action. In particular, we investigated which signaling networks are important for RITA-mediated cancer cell killing, characterized p53/RITA interaction and studied the effect of RITA on mutant p53.

We previously demonstrated that transactivation of pro-apoptotic genes is required for cell death induced by RITA-reactivated p53 (Enge et al., 2009). We found that the activation of pro-apoptotic targets is not sufficient for a full-scale induction of cell death by p53. Here, we showed that a dramatic and rapid downregulation of a number of critical oncogenes and oncogenic pathways by RITA-reactivated p53 is required for the induction of apoptosis. Importantly, our results indicate that induction of pro-apoptotic genes and inhibition of anti-apoptotic/survival genes represent two branches of p53 response, which are differentially regulated. Our results suggest that p53-mediated transrepression is more tightly controlled than transactivation and correlates with increased p53 and reduced Mdm2 abundance on chromatin.

To address the molecular mechanism through which RITA interferes with Mdm2, we mapped RITA-binding site in p53 using a series of deletion and point mutants. We found that RITA binds outside of the Mdm2 binding site and identified S33 and S37 as key p53 residues targeted by RITA. This implies that p53/Mdm2 interaction is prevented by RITA via allosteric mechanism. We propose that RITA/p53 binding induces a conformational trap locking Mdm2-contacting residues in an orientation unfavorable for the p53/Mdm2 binding. Moreover, we found that the conformational change induced by RITA prevents the binding of another p53 inhibitor, MdmX.

Half of human tumors carry point mutations in the p53 gene that abolish p53 binding to DNA. This correlates with poor prognosis and often confers increased resistance to conventional chemo- and radiotherapy. The ability of RITA to induce a conformational change in p53 prompted us to test whether RITA can also restore mutant p53 activity. Here, we found that RITA suppressed the growth and induced apoptosis in a p53-dependent manner in a variety of cell lines that harbor different hot spot p53 mutations. Several known p53 target genes changed their expression in mutant p53-expressing cells upon RITA treatment. Inhibition of mutant p53 prevented RITA-induced effects, suggesting the observed transcriptional response and cell death are dependent on mutant p53.

In summary, our findings demonstrate, that p53 reactivated by the small molecule RITA induces ablation of oncogenic pathways crucial for the survival of cancer cells. RITA acts via an allosteric mechanism and restores the function of both wt and mutant p53.

LIST OF PUBLICATIONS

- I. **V. V. Grinkevich**, F. Nikulenkov, Y. Shi, M. Enge, W. Bao, A. Maljukova, O. Sangfelt, A. Gluch, A. Kel, and G. Selivanova. Ablation of key oncogenic pathways by RITA-reactivated p53 is required for efficient apoptosis of tumor cells. *Cancer Cell* 15 (2009) May 5, 441-453.
- II. J. Zawacka-Pankau, **V. V. Grinkevich**, A. Vema, K. Ridderstråle, E. Hedström, C. Spinnler, V. Andreotti, A. Inga, L-G. Larsson, A. Pramanik, A. Karlén, A. L. Okorokov, and G. Selivanova. Targeting p53 residues S33 and S37 by RITA inhibits p53/Mdm2 and p53/MDMX interactions via an allosteric mechanism. **Manuscript**
- III. C. Y. Zhao, **V. V. Grinkevich**, F. Nikulenkov, W. Bao and G. Selivanova. Rescue of the apoptotic- inducing function of mutant p53 by small molecule RITA. *Cell Cycle*. (2010) May;9(9):1847-55.

ASSOCIATED PUBLICATIONS

A. L. Okorokov, M.B. Sherman, C. Plisson, **V. Grinkevich**, K. Sigmundsson, G. Selivanova, J. Milner and E. V. Orlova. The structure of p53 tumour suppressor protein reveals the basis for its functional plasticity. *EMBO J.* (2006) Nov 1; 25(21), 5191-200.

V.V. Grinkevich, N. Issaeva, S. Hossain, A. Pramanik and G. Selivanova (2005) Reply to 'NMR indicates that the small molecule RITA does not block p53-Mdm2 binding in vitro' *Nature Medicine* 11, 1136-1137.

J. Zawacka-Pankau[#], **V. V. Grinkevich**[#], S. Hüntten, A. Gluch, M. Enge, A. Kel and G. Selivanova. The p53-dependent inhibition of ATP-generating pathways in cancer cells by small molecule RITA converges on the first steps of glycolysis. ([#] shared authorship) **Submitted**

V. V. Grinkevich[#], A. Warnecke[#], C. Spinnler, A. Gluch, A. Kel, K. Pokrovskaja and G. Selivanova. Abrogation of STAT3 pathway in tumor cells by pharmacologically activated p53 ([#] shared authorship) **Manuscript**

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LIST OF ABBREVIATIONS

| | |
|-------------|---|
| ARF | Alternative reading frame |
| ATM | Ataxia telangiectasia mutated |
| CDKs | Cyclin dependent Kinases |
| DBD | DNA binding domain |
| DNA | Deoxyribonucleic acid |
| EGFR | Epidermal growth factor receptor |
| FBW7 | F-box and WD repeat domain-containing 7 |
| FCS | Fluorescence correlation spectroscopy |
| GADD45 | Growth arrest and DNA damage gene 45 |
| GSK3 | glycogen synthase kinase 3 |
| GST | Gluthathione-S-transferase |
| HIPK2 | Homeodomain-interacting protein kinase 2 |
| HPV | Human papilloma virus |
| IGF1R | Insulin-like growth factor 1 receptor |
| IGF1R | Insulin-like growth factor 1 receptor |
| IRS | Insulin receptor substrate |
| MAPK | Mitogen activated protein kinase |
| Mdm2 | Murine double Minute-2 |
| mt | Mitochondrial |
| mTOR | Mammalian target of rapamycin |
| mutp53 | Mutant p53 |
| Myc | Myelomatosis |
| PI3K | Phosphatidyl inositol-3 kinase |
| PTEN | Phosphatase and tensin homolog |
| Puma | P53 upregulated modulator of apoptosis |
| RITA | Reactivation of p53 and induction of tumor cell apoptosis |
| RNA | Ribonucleic Acid |
| ROS | Reactive oxygen species |
| shRNA | Short hairpin RNA |
| siRNA | Small interfering RNA |
| TIGAR | <i>TP53</i> -inducible glycolysis and apoptosis regulator |
| <i>TP53</i> | Tumor protein p53 |
| WHO | World Health Organization |
| wtp53 | Wild type p53 |

1. INTRODUCTION

1.1 CANCER

According to the World Health Organization (WHO), cancer is a leading cause of death worldwide. In Europe alone there are estimated 3.2 million new cases each year (53% occurring in men, 47% in women) and 1.7 million deaths (56% in men, 44% in women). The increased life expectancy of the population will cause these numbers to continue to grow even if age-specific rates remain constant. The most common form of cancers is breast cancer (13.5% of all cancer cases), followed by colorectal cancer (12.9%) and lung cancer (2.1%). However, lung cancer is the most common cause of death, followed by colorectal, breast and stomach cancers (Ferlay et al., 2007).

The development of cancer is a multistep process during which normal cells transform into malignant cells through a series of genetic alterations. Ten hallmarks of cancer, as recently described, include: sustained proliferative signaling, escape from growth suppressors, avoidance of immune destruction, enabled replicative immortality, tumor-promoting inflammation, activation of invasion and metastasis, induction of angiogenesis, genome instability and mutations, resistance to cell death and deregulation of cellular energetics (Hanahan and Weinberg, 2011). The genes implicated in tumorigenesis can be roughly classified in two categories, oncogenes (e.g., c-Myc, mutant Ras, etc) and tumor suppressors (p53, Rb, APC, etc).

1.2 TUMOR SUPPRESSOR P53

The p53 gene (*TP53*) was first described in 1979 and was originally believed to be an oncogene (Lane and Crawford, 1979; Linzer and Levine, 1979). It was only ten years later that its true function as a tumor suppressor gene was discovered (Finlay et al., 1989). In the majority, if not all tumors, p53 function is defective, either due to the p53 gene mutations (50% of tumors) or via overexpression of its negative regulators, mainly Mdm2 (murine double minute 2; HDM2 in humans) (Figure 1 and 2). p53 - “the guardian of the genome” (Lane, 1992) - plays an important role in controlling cell life and death by regulating cell cycle arrest, apoptosis, DNA repair and senescence (Levine et al., 2006).

1.2.1 Regulation of p53

In normal cells the *TP53* gene is continuously transcribed and translated but the protein is rapidly subjected to ubiquitin-dependent degradation in proteasomes. The inhibition of p53 function is performed primarily by Mdm2 (Figure 1): it associates with the N-terminal part of p53 and inhibits its transcriptional function (Momand et al., 1992). In addition to that, Mdm2 acts as an E3-ubiquitin ligase, targeting p53 for proteasomal degradation (Haupt et al., 1997; Kubbutat et al., 1997). This involves interactions with the p53 core and C-terminus, in addition to the N-terminal site (Poyurovsky et al., 2010; Wawrzynow et al., 2009). Mdm2 can also promote NEDD8 modification of p53, which inhibits p53 transcriptional activity (Xirodimas et al., 2004). In response to a number of stress signals including DNA damage, oncogene activation and hypoxia, p53 is stabilized and activated via a series of post-translational modifications, such as phosphorylation or acetylation (Giaccia and Kastan, 1998).

Since the promoter of *MDM2* gene contains a p53-binding-site and is regulated in a p53-dependent manner, Mdm2 and p53 form an autoregulatory feedback loop (Wu et al., 1993) (Figure 1). Upon oncogene activation p14ARF, an antagonist of Mdm2, facilitates p53 activation (Sherr and Weber, 2000). In cancers harboring wt p53 protein, Mdm2 is often overexpressed or hyperactivated through multiple mechanisms (Figure 2). A plethora of modifications and interaction partners that modulate p53's abundance and activity have been identified and new ones are continuously discovered (reviewed in Boehme and Blattner, 2009). In addition to Mdm2, several other E3-ligases for p53 have been identified, such as COP1 (Dornan et al., 2004) and PIRH2 (Leng et al., 2003).

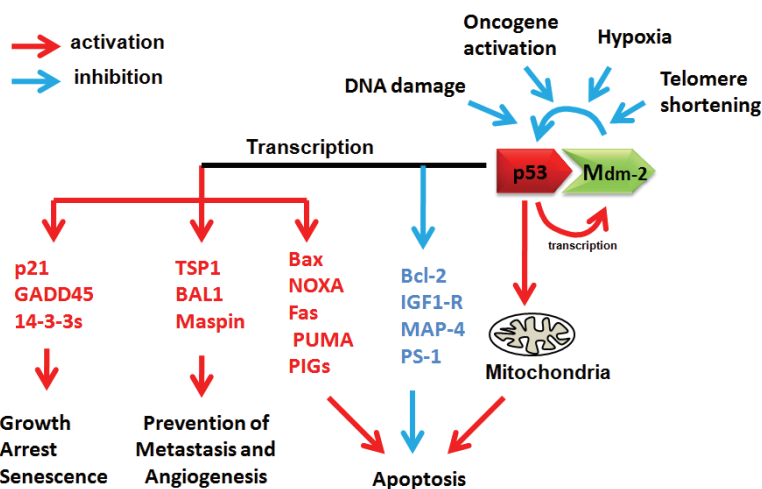


Figure 1. In response to different stress signals p53 becomes stabilized and activated and induces different biological responses through transcriptional activation and transrepression of its target genes or through direct translocation to mitochondria.

1.2.2 Functional roles of p53

p53 acts primarily as a transcription factor. The human p53 protein is 393 amino acid residues long and consists of three major domains: N-terminal transactivation domain, core DNA-binding domain (DBD) and C-terminal oligomerization domain. In response to different stress signals, p53 is activated and induces different cellular responses through transcriptional activation or repression of its target genes (Figure 2). p53 is one of the most highly connected nodes in the cell (Vogelstein et al., 2000). Several dozen genes that are controlled directly by p53 have been identified (el-Deiry, 1998). p53 target genes are involved in a number of cellular processes, the major ones include: cell cycle inhibition, regulation of genomic stability, regulation of redox state and induction of apoptosis.

Cell-cycle inhibition

The gene *CDKN1A*, coding for the protein p21/WAF1, is probably the best known cell cycle inhibitor that is directly targeted by p53. p21 inhibits cyclin-dependent kinases (CDKs) - the key regulators of the cell cycle - and thus blocks the transition from G1 to S phase (Harper et al.,

1993), as well as from G2 to M (Bunz et al., 1998). Two other examples of the p53 targets regulating cell cycle are GADD45 (Growth arrest and DNA damage inducible gene 4) (Wang et al., 1999) and 14-3-3 σ (Chan et al., 1999).

Regulation of genetic stability

p53 plays an important role in maintaining genetic stability. It is involved in the induction of genes that regulate nucleotide excision repair, base excision repair and homologous recombination (reviewed in Sengupta and Harris, 2005). Recently, p53 has been reported to play a novel role in maintaining mitochondrial genetic stability via direct interaction with mitochondrial (mt) DNA polymerase γ (pol γ) and enhancement of its function in response to mtDNA damage induced by exogenous and endogenous effectors including ROS (Achanta et al., 2005). Furthermore, upon DNA damage p53 induces the expression of the ribonucleotide reductase (*RRM2B*), a rate-limiting factor in DNA repair (Tanaka et al., 2000), p48 (*DDB2*) and xeroderma pigmentosum gene (*XPC NER*) (Fitch et al., 2003).

Redox control

Through its target genes such as TIGAR (*TP53*-inducible glycolysis and apoptosis regulator) and sestrins, p53 can downregulate the level of reactive oxygen species (ROS) to prevent the accumulation of daily DNA damage (Budanov et al., 2004; Sablina et al., 2005). In the case of extended stress and irreparable damage, p53 upregulates ROS level causing apoptosis via induction of PIG's (Bensaad and Vousden, 2005).

Apoptosis

p53 is a potent inducer of apoptosis due to its ability to transactivate pro-apoptotic target genes. The pro-apoptotic protein Fas is a transmembrane protein (Owen-Schaub et al., 1995) that trimerizes upon Fas ligand binding and induces the apoptotic cascade. Other pro-apoptotic proteins like Bax (Miyashita and Reed, 1995), Puma (Nakano and Vousden, 2001) or Noxa (Oda et al., 2000) are localized at the mitochondria, promoting the release of cytochrome c into the cytoplasm and consequently launching the apoptotic cascade by activating caspases. In addition, p53 can promote apoptosis through transcriptional repression of survival factors and anti-apoptotic genes (e.g. Bcl-2 and survivin) or through direct localization to mitochondria (Marchenko et al., 2000) and (reviewed in Vaseva and Moll, 2009) (Figure 1).

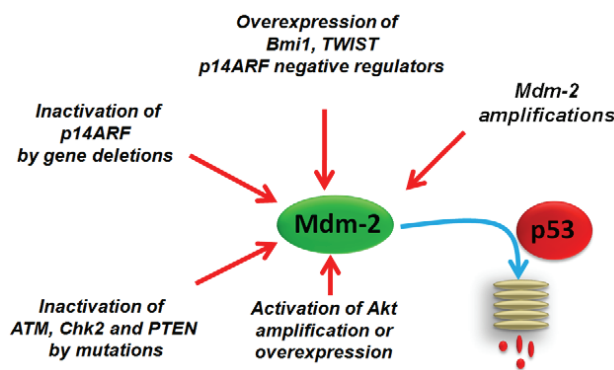


Figure 2. Different pathways of Mdm2 hyperactivation in cancers

14-3-3 pathways. In addition, IGF-1R has been shown to be crucial for anchorage-independent growth (Baserga, 2000). This feature is unique to cancer cells; the degree of anchorage independency reflects the level of malignancy. Several studies, both experimental and clinical, have demonstrated that the IGF-1R is overexpressed in cancer cells and high plasma levels of IGFs is a potential risk factor (reviewed in Larsson et al., 2005). The overexpression of IGF-1R in neoplastic cells and its crucial role in cancer cell growth makes this tyrosine kinase receptor an attractive target for anti-cancer therapy. Inhibition of IGF-1R causes massive apoptosis of tumor cells *in vivo*, and blocks tumor invasion and metastasis (reviewed in Larsson et al., 2005). Both p53 and Mdm2 have been shown to be involved in the regulation of IGF-1R; while p53 can repress its transcription (Werner et al., 1996) Mdm2 is involved in its proteasomal degradation (Gimita et al., 2003).

1.3.2 Signal transducers - PI3-kinase pathway (PI3K, Akt, mTOR and GSK3 β)

PI3-kinase

Phosphoinositide 3-kinase (PI3K) plays an important role in a plethora of biological processes, which include several hallmarks of cancer described by Hanahan and Weinberg, i.e., the increased proliferation, survival, metabolism, invasion and angiogenesis. PI3K has two functional subunits, ~100 kDa catalytic subunit p110 (one of α , β or δ) and a non-catalytic regulatory subunit p85 (one of α , β or γ) (reviewed in Stokoe, 2005). Activation of the PI3-kinases is induced by a number of growth factors and cytokines and results in phosphorylation of phospholipid substrates - phosphatidylinositols (PtdIns). Upon activation, p85 subunit which associates with activated tyrosine kinase receptor, attracts p110 subunit. PI3-kinase heterodimer translocates to the plasma membrane to its substrate phospholipids (reviewed in Stokoe, 2005). Activators of PI3-kinase signaling include the platelet-derived growth factor receptor (PDGF), epidermal-growth factor receptor (EGFR/HER1), HER3, the stem cell factor receptor c-Kit, the tyrosine protein kinase Abl, polyoma virus middle T antigen and IRS proteins. Tumor suppressor p53 can regulate PI3K activity on several levels. p53 is a transcriptional repressor for *PIK3CA* gene encoding the p110 α subunit (Astanehe et al., 2008) and it is a transcriptional activator of *PTEN*, the negative regulator of PI3K (Stambolic et al., 2001). Alterations of PI3K's occur in human tumors with high frequency, including activating mutations in the *PIK3CA* gene (p110 α), *PIK3R1* gene (p85 α) and amplifications of *PIK3CB* (p110 β) (Liu et al., 2009).

Akt kinase

The major PI3-kinase downstream target is the serine/threonine protein kinase B (PKB, also termed Akt). Following PI3-kinase activation, Akt translocates to the plasma membrane and binds to the activated PtdIns (Figure 3). At the membrane, Thr308 in the activation loop of Akt is phosphorylated by a protein kinase termed phosphoinositide-dependent kinase 1 (PDK-1) (Stokoe, 2005). Akt is also phosphorylated on Ser473, most probably by mTOR (mammalian target of rapamycin) and DNA-PK (DNA-dependent protein kinase), resulting in the stabilization of its active conformation (Yang et al., 2002). Akt mediates some of the biological processes attributed to PI3-kinase activity that are important in tumorigenesis, for example regulation of substrates playing a role in cell survival, including Foxo transcription factors, glycogen synthase kinase 3 (GSK3) (Pap and Cooper, 1998) and Mdm2. Other known

Akt substrates comprise Bad, IKK and caspase-9. Amplification of *AKT* gene and activating mutation in the pleckstrin homology domain of Akt (E17K) have been reported in various tumor types (Liu et al., 2009).

mTOR-kinase

mTOR is a downstream target of Akt. One of the mechanisms of mTOR activation is a direct phosphorylation of its Ser2448 by Akt (Stokoe, 2005). The major function of mTOR is the positive regulation of mRNA translation. The best characterized substrate of mTOR mediating this translation is 4EBP1, an inhibitor of the translation initiation factor eIF4E. Phosphorylation of 4EBP1 by mTOR releases it from eIF4E allowing eIF4E to recruit mRNAs to the ribosome (Hay and Sonenberg, 2004).

Translation factor - oncogene eIF4E

Oncogenic Ras and Akt signal transduction pathways through mTOR kinase converge on a crucial effector of translation, the initiation factor eIF4E, which binds the 5' cap of mRNAs (Gingras et al., 1999). In eukaryotes, most mRNAs are translated in a cap-dependent manner. The cap structure m⁷GpppN (where N is any nucleotide) is found at the 5' end of all cellular eukaryotic mRNAs. An alternative mechanism of translation initiation is cap- and eIF4E-independent and requires an internal RNA structure termed Internal Ribosome Entry Site (IRES) to which the 40S ribosomal subunit binds directly. eIF4E controls the translation of various malignancy-associated mRNAs which are involved in polyamine synthesis, cell cycle progression, activation of proto-oncogenes, angiogenesis, autocrine growth stimulation, cell survival, invasion and communication with the extracellular environment (reviewed in Mamane et al., 2004). As eIF4E also associates with RNA helicases, mRNAs containing long and structured 5' untranslated regions (such as cyclin D and c-Myc mRNAs) are particularly sensitive to eIF4E deregulation. eIF4E is overexpressed in several human cancers including cancers of the colon, breast, bladder, lung, prostate, gastrointestinal tract, head and neck, Hodgkin's lymphomas and neuroblastomas (Mamane et al., 2004). Moreover, eIF4E cooperates with v-Myc and E1A to immortalize rat embryo fibroblasts, while tumor suppressor p53 has been shown to be a negative transcriptional regulator of *EIF4E* (Zhu et al., 2005). Reduction of eIF4E expression leads to a decrease in global translation rates, a reversal of the transformed morphology and decreased growth in soft agar (De Benedetti et al., 1991; Rinker-Schaeffer et al., 1993). Taken together, these features of eIF4E make it an attractive target for anticancer therapy.

GSK3 β -kinase and tumor suppressor FBW7

Glycogen synthase kinase β is one of the Akt substrates. GSK3 β has been discovered nearly three decades ago in rabbit skeletal muscle as a protein kinase that phosphorylates and inactivates glycogen synthase, the final enzyme of glycogen biosynthesis (reviewed in Mishra, 2010). GSK3 β is a multifunctional Ser/Thr kinase with diverse roles in various human diseases, including diabetes, inflammation, neurological disorders and various neoplastic diseases. Until now, two members of the mammalian GSK3 family, α and β , have been described. GSK3 β activity is regulated by a site-specific phosphorylation of Tyr216 and Ser9 residues. GSK3 β is constitutively active in quiescent cells and undergoes a rapid and transient inhibition in response to a number of external signals. Many upstream kinases, such as protein kinase A, Akt, protein kinase C and others can phosphorylate Ser9 of GSK3 β and thus inactivate it. The role of GSK3 β in cancer appears to be context-dependent: it has a growth-

promoting role in some cancers, whereas it suppresses growth in others (Mishra, 2010). Phosphorylation by GSK3 β regulates the stability of various oncogenic transcription factors like AP-1, NF κ B, c-Myc, β -catenin, Snail, Forkhead, C/EBP and CREB by triggering their proteasomal degradation (Mishra, 2010). Moreover, GSK3 β can directly bind to the tumor suppressor p53 and promote its phosphorylation and acetylation; however, the functional consequences of this interaction remain controversial (Eom and Jope, 2009; Pluquet et al., 2005; Watcharasit et al., 2002).

Phosphorylation of substrates by GSK3 β is followed by degradation mediated by FBW7, at least in several cases. FBW7 (F-box and WD repeat domain-containing 7) is the substrate recognition component of an SCF (complex of SKP1, CUL1 and F-box protein)-type ubiquitin ligase. FBW7 is a tumor suppressor the regulatory network of which is perturbed in many human malignancies. It mediates the degradation of several proto-oncogenes that function in cellular growth and division pathways, including c-Myc, cyclin E, β -catenin, Notch and c-Jun. Moreover, recently FBW7 has been found to negatively regulate stability of another potent oncogene Mcl-1, which depends on GSK3 β phosphorylation (Inuzuka et al., 2011). In tumors with loss of FBW7 the level of Mcl-1 is considerably increased (Wertz et al., 2011). The inactivation of FBW7 function by mutations or deletions causes chromosomal instability and is associated with tumorigenesis (Welcker and Clurman, 2008).

1.3.1 Transcription factor - oncogene c-Myc

c-Myc is one of the most widely studied proto-oncogenes. The *MYC* gene is localized to chromosome 8 in a region that is translocated in Burkitt's lymphomas (Dalla-Favera et al., 1982). c-Myc is a short-lived transcription factor with a half-life of 20-30 min. Together with its partner Max it binds DNA and transactivates its target promoters. c-Myc regulates a broad range of genes which are involved in cell cycle, proliferation, ribosomal biogenesis and metabolism, differentiation, apoptosis, transformation, genomic instability and angiogenesis (Oster et al., 2002). By combining chromatin immunoprecipitation (ChIP) and promoter microarrays, researchers recently identified 1469 direct target genes of c-Myc. Interestingly, p53-activated genes are quite often repressed by c-Myc, for example p21, Mdm2, GADD45 and others (Ceballos et al., 2005).

Regulation of c-Myc expression and activity occurs on multiple levels through transcriptional, posttranscriptional, translational, and posttranslational mechanisms. On the transcriptional level, *MYC* gene is regulated by signal transduction pathways that are often deregulated in cancer. Tumor suppressor p53 has been shown to be a transrepressor of *MYC* (Ho et al., 2005). At the posttranslational level the c-Myc protein stability is controlled through sequential and reversible phosphorylation at two highly conserved sites, threonine 58 and serine 62 (Hann, 2006). GSK-3 β mediated phosphorylation of c-Myc at threonine 58 allows for binding of the ubiquitin ligase FBW7 and recruitment of the SCF complex followed by proteasomal degradation (Dai et al., 2006). The majority of human cancers display deregulated c-Myc activity (Ponzielli et al., 2005). In addition to mutations in the upstream signalling, increased expression of c-Myc is achieved via chromosomal translocations exemplified by the c-Myc-Immunoglobulin fusion gene in Burkitt's lymphoma, *MYC* gene amplification, as well as mutations in the gene leading to the stabilization of the c-Myc protein (reviewed in Albiñan et al., 2010).

1.3.1 Apoptosis regulators – oncogenes Bcl-2, Mcl-1 and survivin

The Bcl-2 family comprises apoptosis regulators with pro- or anti-apoptotic function (Altieri, 2003). Via regulation of mitochondrial permeability and release of cytochrome c, anti-apoptotic Bcl-2 family members Bcl-2, Mcl-1 and Bcl-XL inhibit apoptosis, whereas pro-apoptotic BAX, BAD, BAK and BID enhance apoptosis. Several models for the function of Bcl-2 family proteins have been suggested, including physical association with mitochondrial channels and the ability to act as pore-forming structures. Bcl-2 molecules form homo- or hetero-dimers at the mitochondrial membrane and differentially recruit pro- or anti-apoptotic family members, thus controlling the balance between cell death and cell survival. Recently discovered Bcl-2 family member Mcl-1 is attracting more and more interest (Thomas et al., 2010).

Mcl-1 transcription is regulated by a number of externally activated and constitutively active transcription factors, notably the signal transducers and activators of transcription (STAT) family (Akgul et al., 2000) and hypoxia-inducible factor 1 α (Liu et al., 2006). Mcl-1 promoter is directly repressed by E2F-1 (Croxtton et al., 2002) and p53 (Pietrzak and Puzianowska-Kuznicka, 2008). In addition to Mcl-1, Bcl-2 (Miyashita et al., 1994) and survivin (Hoffman et al., 2002) are well established p53 transrepression targets.

Survivin belongs to another family that regulates cell death, the inhibitors of apoptosis (IAP). XIAP, c-IAP1, and c-IAP2 proteins also belong to this family. IAPs counteract apoptosis by acting downstream of Bcl-2 as endogenous inhibitors of caspases. Dramatic overexpression of survivin has been observed in cancers of lung, breast, colon, stomach, oesophagus, pancreas, liver, uterus, ovaries, Hodgkin's disease, non-Hodgkin's lymphoma, leukaemias, neuroblastoma, pheochromocytoma, soft-tissue sarcomas, gliomas and melanoma (reviewed in Altieri, 2003).

1.4 TARGETING ONCOGENE ADDICTION

“Oncogene addiction,” i.e., the dependency of tumor cells on the oncogenic activity that initially contributed to tumor phenotype, first coined by B. Weinstein (Weinstein, 2002), potentially reveals an “Achilles' heel” of cancer cells. This idea is supported by *in vivo* experiments in mice with regulatable c-Myc (Felsher and Bishop, 1999; Pelengaris et al., 2002), BCR-ABL (Huettner et al., 2000), H-ras (Chin et al., 1999), Bcl-2 (Letai et al., 2004) and HER2/NEU (Moody et al., 2002). Targeting “oncogene addiction” is currently a major strategy for the development of novel anticancer drugs.

The fact that many components of the PI3K pathway are frequently mutated or altered in common human cancers and that PI3-kinase pathway is hyperactivated in the majority of tumors is intensively pursued by many pharmaceutical companies. Numerous PI3K, Akt and mTOR - targeting compounds have been and are currently being tested in clinical trials (summarized in Liu et al., 2009). Small-molecule inhibitors of PI3-kinase activity were first described 17 years ago. Wortmannin (Arcaro and Wymann, 1993) is a fungal metabolite that binds covalently to all p110 isoforms of PI3K. LY294002 (Vlahos et al., 1994) is a morpholino derivative of the kinase inhibitor quercetin. However, both wortmannin and LY294002 also inhibit many other protein kinases, related and not related to PI3K, including

ATM, ATR, DNA-PK and mTOR. Interestingly, treatment with wortmannin or LY294002 have shown effective antitumor activity in mouse models, with very little toxicity despite of their broad mechanism of action (Stokoe, 2005). In addition, LY294002 and wortmannin sensitize tumors to conventional chemotherapeutic agents, including bleomycin, vincristine, paclitaxel, as well as novel agents such as immunotoxins and the EGFR inhibitor gefitinib. Second-generation of PI3K inhibitors that are more selective for individual PI3-kinase isoforms are currently being developed by different companies.

PI3K upstream regulators, such as IGF-1R are also promising targets for cancer treatment. Strategies to downregulate IGF-1R or inhibit its TK activity have been associated with the strongest antitumor efficacies (Baserga et al., 2003). A variety of approaches aimed at targeting IGF-1R have been utilized for the development of anticancer therapies; these include the use of antibodies, small interfering RNA's and chemicals (Larsson et al., 2005).

Small-molecule inhibitors of PI3-kinase effectors have also been recently developed. At least two different classes of Akt inhibitors have been described. Phospholipid analogues bind Akt and prevent its translocation to the plasma membrane (aka D-21266 (Phase I clinical trials), PX-316 and the PIA-24). The second class of Akt inhibitors prevents the phosphorylation of Akt by PDK-1. These compounds are highly selective between Akt isoforms and are inactive against closely related protein kinases. Rapamycin, the most studied mTOR inhibitor, was initially considered to be an antifungal agent, then an immunosuppressant (sirolimus/rapamune; approved in the USA in 1999). Rapamycin has been shown to have an anti-tumor activity over 25 years ago (Eng et al., 1984), but its analogues have been tested in clinical trials only recently. CCI-779 (developed by Wyeth-Ayerst), AP23573 (developed by Ariad) and RAD001 (developed by Novartis) have shown efficacy in several human tumor cell lines *in vitro*, and in human and murine tumors *in vivo* (Stokoe, 2005).

The inhibition of c-Myc is one of the promising approaches for anticancer therapies; recent studies in an elegant mouse model, with regulatable c-Myc, provide further support to this idea (Soucek et al., 2008). Molecular screens for compounds interfering with transactivation by c-Myc or with Myc/Max heterodimerization yielded several interesting candidate compounds awaiting further investigation (reviewed in Albihn et al., 2010).

Overexpression of anti-apoptotic Bcl-2 family members has been associated with chemotherapy resistance in various human cancers, and preclinical studies have shown that agents targeting anti-apoptotic Bcl-2 family members have anti-tumor activity as single agents and in combination with other antineoplastic agents. Clinical trials of several investigational drugs targeting the Bcl-2 family (oblimersen sodium, AT-101, ABT-263, GX15-070) are ongoing (Kang and Reynolds, 2009).

Thus, targeting oncogenes addiction is currently a major strategy for the development of novel anticancer drugs. However, even when tumors initially respond to targeted oncogene inactivation, most eventually recur (Giuriato and Felsner, 2003), indicating that alternative approaches are required.

1.5 P53 AS ANTICANCER TARGET

Diverse oncogenic events result in p53 activation and induction of p53-dependent programmed cell death or senescence. These include activation of oncogenes Ras, c-Myc, β -catenin, cdk4 or cyclins D/E, or inactivation of tumor suppressors p16, Rb, or APC and most probably others.

Therefore, it appears that only those tumor cells, which acquired additional mutations blocking p53 response, will survive and continue to expand. Mutations that block apoptosis induction happen frequently in tumors; however, inactivation of p53 function is probably the most efficient way to disable p53-induced response.

In cancer cells, inactivation of p53 is achieved through mutations causing its inability to bind properly DNA or through over-activation or over-abundance of its negative regulators. Therapies aimed to restore the function of inactivated p53 in tumors will re-establish the missing link between oncogene-induced pro-apoptotic signaling and cell death machinery. This should trigger massive apoptosis in pre-sensitized tumor cells leaving normal cells largely unaffected.

The idea that reactivation of the tumor-suppressor protein p53 might help to combat cancer is gaining more and more support. Recent studies in mice with “switchable” p53 demonstrated that restoration of p53 function leads to the suppression of already established tumors - lymphomas, soft tissue sarcomas and hepatocellular carcinomas (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). However, new evidence suggests it is unlikely that all tumors or all cells within the tumor will respond to p53 reactivation. Two independent groups found that p53 restoration resulted in a significant decrease of high-grade tumors, but not low-grade ones (Feldser et al., 2010; Junttila et al., 2010). This selectivity of p53-mediated tumor suppression might lead to incomplete tumor regression of early lesions. On other hand, human tumors usually are already advanced upon detection and are in a state of high oncogenic flux. Advanced tumors might still contain cells with a low oncogenic flux from the earlier lesions; however, they are most likely to constitute only a small fraction of the tumors (see Berns, 2010). Therefore restoring p53 activity should still have a considerable effect on human tumors. The important conclusion from animal studies is that the developed tumors remain vulnerable to p53 restoration.

In summary, p53 is a good target for anticancer therapies due to the following reasons:

1. The high potency of p53-mediated tumor suppression, including irreversible growth suppression and induction of apoptosis, inhibition of tumor stroma and angiogenesis, as well as the block of invasion and metastasis.
2. p53 is inactivated in the majority of tumors and its inactivation is required for the maintenance of tumors.
3. The p53 protein is usually expressed in tumors; the deletions of the p53 gene are very rare, in contrast to other tumor suppressors, such as Rb and p16.

1.6 PHARMACOLOGICAL REACTIVATION OF WTP53

In the recent years the idea of pharmacological reactivation of p53 has been attracting more and more interest, both in academia and in industry. Introducing functional p53 into tumors using adenovirus vectors lead to a success in clinical trials (Edelman and Nemunaitis, 2003) and p53 gene therapy has been approved in China in 2003 (Lane et al., 2010). There are 151 ongoing clinical trials involving p53, as listed in National Cancer Institute database (Cheok et al., 2011). Until now, pharmacological activation of p53 was achieved mainly through treatments with highly genotoxic compounds, like DNA damaging drugs, which do not have selectivity for tumor cells and thus cause dramatic side effects. Therefore, novel therapies based on selective restoration of the p53 tumor suppressor activity in cancer cells without causing genotoxic stress are of great importance.

Since half of the tumors retain functionally inactive wild type p53, and the rest carry the mutated one, two major strategies are currently being pursued: reactivation of wtp53 and restoration of mutant p53 function.

Targeting p53 interaction with its negative regulators as a main strategy to reactivate wt p53

A number of p53-reactivating molecules have been identified during the recent years, both via rational design or random screens of chemical libraries, including biochemical screens *in vitro*, or cell-based phenotypic ones (Table 1). Small molecules have been identified which disrupt p53's interaction with its negative regulators such as Mdm2, MdmX, E6 and others, or compounds inhibiting other p53 negative regulators, such as sirtuins (reviewed in Lane et al., 2010). Molecules that reactivate wtp53 have been shown to elevate p53 protein level and to restore its transcriptional activity, resulting in the induction of apoptosis or growth arrest in tumor cells, in most cases without drastic effects in normal tissues.

| Name | Effect on p53 | | Target | Mechanism of action | Effect <i>in vitro</i> / <i>in vivo</i> | Method of Identification | Reference |
|--------------------------------------|---------------|-----|--------------------------|--|--|---|---|
| | wt | mut | | | | | |
| Mdm2-binding molecules | | | | | | | |
| Nutlin | + | - | Mdm2, p53 binding domain | Prevents p53/Mdm2 interaction | Growth arrest or apoptosis/ tumor suppression | In vitro screen for Mdm2 binding | Vassilev et al., 2004 |
| M1219 | + | - | Mdm2, p53 binding domain | Prevents p53/Mdm2 interaction | Growth arrest or apoptosis/ tumor suppression | Rational design | Shangary et al., 2008 |
| HU98 | + | ND | Mdm2 | Inhibits Mdm2 E3-ligase activity | Apoptosis/ND | In vitro screen for the inhibition of MDM2 autoubiquitination | Yang et al., 2005 |
| SAH-p53 peptide | + | ND | Mdm2, p53 binding domain | Prevents p53/Mdm2 interaction | Apoptosis/ND | Rational design | Bernal et al., 2007 |
| MdmX-binding molecules | | | | | | | |
| SJ172550 | + | ND | MdmX | Prevents p53/MdmX interaction | Apoptosis/ND | Biochemical and cell-based screen | Reed et al., 2010 |
| Stapled peptide SAH-p53-8 | + | + | MdmX | Prevents p53/MdmX and p53/Mdm2 interaction | Apoptosis/ tumor suppression | Rational design | Bernal et al., 2010 |
| p53-binding molecules | | | | | | | |
| RITA | + | + | p53, N-terminus | Prevents p53/ Mdm2 interaction | Apoptosis/ tumor suppression | Cell-based screen of chemical library | Issaeva et al., 2004 |
| SCH529074 | + | + | p53, DNA-binding domain | Restores wt p53 conformation | Apoptosis/ND | In vitro screen for p53 DNA binding of quinazolines | Demma et al., 2010 |
| PRIMA-1 ^{MT} /APR-246 | + | + | p53 | Restores wt p53 conformation | Apoptosis/ tumor suppression | Cell-based screen of chemical library | Bykov et al., 2002 |
| Peptide CDB3 | + | + | p53, DNA-binding domain | Restores wt p53 conformation | Weak apoptosis/ND | Rational design | Friedler et al., 2002. Issaeva et al., 2002 |
| CP31398 | + | + | p53, DNA-binding domain | Restores wt p53 conformation | Apoptosis/ND | In vitro screen | Foster et al., 1999 |
| Other p53 negative regulators | | | | | | | |
| Tenovin | + | + | Sirtuin 1 and 2 | Induces p53 activating acetylation | Apoptosis/ tumor suppression | Luciferase reporter screen | Lain et al., 2008 |

Table 1. Molecules restoring p53 function via targeting p53 or p53-binding proteins

* effect is observed in the certain cancer cell lines

Targeting Mdm2 as the most promising strategy to reactivate wt p53

The major negative regulator of p53 is E3 ligase Mdm2, which ubiquitinates p53 and promotes its translocation to the cytoplasm, thus leading to p53 degradation by proteasomes. In addition, Mdm2 can inhibit p53 transcriptional activity at the promoters (Momand et al., 1992). Given the central role of Mdm2 in the control of p53 function, the potential for non-genotoxic cancer therapies based on the inhibition of Mdm2 is considerable. Different strategies of Mdm2 inhibition could be envisioned, including the inhibition of Mdm2 E3 ligase activity or p53/Mdm2 interaction, block of Mdm2 synthesis by antisense oligonucleotides or small interfering RNAs, or inhibition of Mdm2 transcription by inhibitors of RNA polymerase II. In addition, Mdm2-induced degradation of p53 can be blocked by proteasomal inhibitors or via prevention of p53 nuclear export by Leptomycin B (Hainaut and Wiman, 2005).

Prevention of p53/Mdm2 interaction appears to be a promising way to restore p53 activity in tumors. The first evidence supporting this concept came from a study performed by Bottger et al. In this study a small p53-based peptide has been designed that binds tightly to the p53-binding pocket in Mdm2; its expression in cells results in p53 activation and induction of growth arrest (Bottger et al., 1997). Another synthetic peptide derived from p53 (AP peptide) inhibits the p53-Mdm2 interaction, induces p53 accumulation and activation followed by apoptosis in tumor cells with overexpressed Mdm2 (Chene et al., 2000). Although peptides are attractive candidates for stabilizing or disrupting protein-protein interactions, their efficacy *in vivo* is severely compromised due to the loss of secondary structure, susceptibility to proteolytic degradation, and inefficient cellular uptake. Synthetic approaches such as hydrocarbon stapling, which reinforce native peptide sequences, provide an alternative strategy to manipulate protein-protein interactions via peptides, as shown using Stabilized Alpha Helix of p53 (SAH-p53) peptide modeled after the transactivation domain of p53. SAH-p53 binds Mdm2 and reactivates the p53 pathway in Mdm2-overexpressing osteosarcoma cells (Bernal et al., 2007).

Protein-protein interactions which constitute the major key nodes in many signaling pathways are usually regarded to be a difficult target for small molecules. However, the identification and development of small molecules-inhibitors of p53/Mdm2 interaction provide an inspiring example.

Several classes of small molecules that can directly bind to Mdm2 and inhibit its interaction with p53 have been successfully identified. Nutlins are a group of cis-imidazoline analogs which mimic the orientation of key residues in the p53 N-terminal alpha-helix, Phe19, Trp23 and Leu26, which are critical for binding to Mdm2 (Linke et al., 2008; Vassilev et al., 2004). Nutlin3a sterically blocks the hydrophobic cleft where p53 binds, displaces p53 from Mdm2 leading to p53 accumulation and restoration of its transcriptional activity (Vassilev et al., 2004). Nutlin3a promotes potent growth inhibition in a number of tumor cell lines of different origin, including colon, lung, breast and skin carcinomas, melanoma, different lymphomas and others and suppresses tumor growth *in vivo* (Cheok et al., 2011).

Another small molecule, MI-219 (also known as AT-219; Ascent Therapeutics, Malvern, Pa, USA) was rationally designed based on the crystal structure of the p53/Mdm2 complex. MI-219 fits in the p53-binding pocket of Mdm2 in the same manner as Nutlin3a, mimicking the key p53 residues interacting with Mdm2. It activates the p53 pathway in wt p53-expressing cancer cells and leads to the induction of cell cycle arrest and, in some cells, apoptosis.

Although MI-219 activates p53 in normal tissues, p53 accumulation is minimal and not harmful to animals. It is a potent, highly selective and orally active compound which can inhibit tumor growth *in vivo* (Shangary et al., 2008). Both Nutlin3a and MI-219 appear not to have a substantial off-target activity.

Furthermore, several compounds targeting p53/Mdm2 interaction are currently undergoing clinical trials: JnJ-26854165 (Johnson & Johnson, USA), PXn727 and PXn822 (Priaxon, Munich, Germany) and RG7112 a compound from the Nutlin3a series (F. Hoffmann–la Roche, Basel, Switzerland) (Cheek et al., 2011).

Targeting MdmX is an essential step for p53 reactivation

MdmX is an Mdm2 homologue, but unlike Mdm2 it does not have E3 ubiquitin ligase activity (Vousden and Lu, 2002). MdmX inhibits p53-mediated transcription via direct interaction with p53. Several studies have highlighted the importance of developing inhibitors towards MdmX. It has been shown that MdmX forms heterodimers with Mdm2 and enhances its E3 ligase activity, therefore MdmX depletion leads to elevated p53 levels (Linares et al., 2003), suppresses colony outgrowth (Danovi et al., 2004) and reduces tumor xenograft growth (Gilkes et al., 2006).

Nutlin3a, which mainly induces growth arrest in cancer cells, has a 30-fold lower efficiency in disrupting the p53/MdmX interaction compared to the p53/Mdm2 complex (Hu et al., 2006; Laurie et al., 2006; Patton et al., 2006; Wade et al., 2006). MI-219, which has biological effects similar to Nutlin3a, is 10,000-fold more selective for Mdm2 over MdmX.

The efficacy of Mdm2 inhibition can be compromised by overexpression of MdmX, while down-regulation of MdmX in combination with Nutlin3a significantly enhances p53 activity (Hu et al., 2006; Patton et al., 2006; Wade et al., 2006; Xia et al., 2008). There have been reports of short peptides inhibiting the interaction between MdmX and p53 (Hu et al., 2007; Pazgier et al., 2009), however the clinical application of peptides is not optimal due to their proteolytic degradation and the insufficient cellular uptake. Recently identified chemical inhibitor of MdmX/p53 binding, SJ-172550, confers an additive growth suppression effect in cancer cells when combined with Mdm2 inhibitors (Reed et al., 2010). In addition, stapled p53 helix peptide (SAH-p53-8) has been designed to preferentially target MdmX (Bernal et al., 2010). It blocks the formation of inhibitory p53-MdmX complexes, induces p53-dependent transcriptional activation of genes and thereby overcomes MdmX-mediated resistance *in vitro* and *in vivo*. Stapled peptides might open the way for peptide-targeted therapies.

Direct targeting of p53

Targeting Mdm2 for restoring p53 activity is currently actively pursued both in academia and industry. In contrast, strategies aimed at direct targeting p53 have not been as popular so far. The major reason is that the p53 protein is conformationally flexible and its N-terminus where Mdm2 binding site resides is considered to be largely unstructured in solution. In fact, the full-length p53 protein eluded numerous attempts of crystallization, largely due to its intrinsic structural flexibility. The absence of the structural data limits the possibility of rational drug design (Selivanova, 2010).

We have identified a small molecule RITA (reactivation of p53 and induction of tumor cell apoptosis) which prevents interaction between p53 and Mdm2 by binding directly to p53 and

inducing a conformational change which prevents the interaction of p53 with Mdm2 and several other p53 inhibitors (Issaeva et al., 2004).

Notably, several p53-binding molecules that reactivate mutant p53 have been shown to activate the function of wt p53 in cancer cells. These include CDB3 (Issaeva et al., 2003), SCH529074 (Demma et al., 2010), CP-31398 (Luu et al., 2002) and PRIMA-1^{MET} (Bao et al., 2011) (Table 1). The mechanism of wild type p53 reactivation by these molecules is not completely understood. At least some of them appear to inhibit the p53/Mdm2 interaction. Since all these molecules restore mutant p53 function via binding p53 and changing its conformation, it is tempting to speculate that the wild type p53 could be reactivated via an allosteric mechanism.

Blocking E6 is a promising strategy to reactivate p53 in cervical cancers

In over 90% of cervical cancers the p53 tumor suppressor pathway is disrupted by human papillomavirus (HPV). HPV-E6 promotes the degradation of wild type p53 by recruiting cellular E3 ligase E6-associated protein (E6AP) thus inhibiting p53 in a manner similar to Mdm2 (Scheffner et al., 1993). Mdm2 plays a minor role in p53 regulation in the presence of E6 (Hengstermann et al., 2001). Prevention of p53 destruction by HPV-E6 appears to be an important strategy to combat cervical carcinoma. Inhibition of HPV-E6 can be achieved by inhibition of E6 by a synthetic peptide ligand (Dymalla et al., 2009), by depleting E6 mRNA, (Goodwin and DiMaio, 2000; Jiang and Milner, 2002; von Knebel Doeberitz et al., 1992) or inhibition of E6 mRNA by the combination of leptomycin B and actinomycin D or by small molecules ChemBridge 5254239 and 5228473 (Hietanen et al., 2000; Kochetkov et al., 2007). In addition, inhibiting E6AP by RNAi or a dominant-negative mutant has been shown to induce p53 (Kelley et al., 2005; Talis et al., 1998). Interestingly, we have found that p53 targeting by RITA prevents its interaction with E6-associated protein, which is required for HPV-E6-mediated degradation, and induces the accumulation of p53 in cells containing high-risk HPV16 and HPV18 (Zhao et al., 2010). In several cervical cancer cell lines RITA activates the transcription of pro-apoptotic p53 targets Noxa, PUMA, and BAX, as well as represses the expression of pro-proliferative factors CyclinB1, CDC2, and CDC25C. This results in p53-dependent apoptosis and cell cycle arrest. Importantly, RITA substantially suppresses the growth of cervical carcinoma xenografts *in vivo* (Zhao et al., 2010).

Inhibition of Sirtuins as a novel strategy to reactivate wt p53

Sirtuins have been shown to be a direct NAD⁺-dependent deacetylases for a variety of proteins involved in diverse cellular processes, such as cellular metabolism, extended cellular life span and differentiation, as well as in negative regulation of p53 (Haigis and Guarente, 2006). Several inhibitors of sirtuin deacetylase activity have been described, including sirtinol, cambinol, and EX-527 (Lain et al., 2008). Tenovin 1 and its more soluble derivative Tenovin 6 are two novel sirtuin inhibitors identified by Lain et al. using a p53-dependent reporter as readout for the screen of chemical library (Lain et al., 2008). Tenovin 1 inhibits Sirtuin 1, thus inducing acetylation of p53 at lysine 382 and activating p53. It suppresses the growth of various tumor cell lines and inhibits the growth of highly aggressive melanoma xenograft tumors without causing DNA damage (Lain et al., 2008).

1.6.1 RITA

RITA (Reactivation of p53 and Induction of Tumor cell Apoptosis) is a low molecular weight compound identified by our group in a cell-based screen for wild-type p53-reactivating molecules (Issaeva et al., 2004). RITA is a 2,5-bis(5-hydroxymethyl-2-thienyl)furan (NCS652287) (Figure 4) which binds the N-terminus of p53 (residues 1-63), inducing a conformational change that prevents p53 from binding to Mdm2, resulting in p53 accumulation and consequently p53 activation and induction of apoptosis in various cancer cell lines.

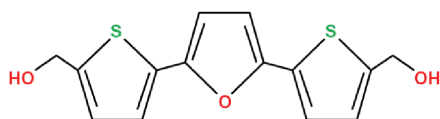


Figure 4 Chemical structure of RITA - 2,5-bis(5-hydroxymethyl-2-thienyl)furan -NCS652287

Furthermore, RITA suppresses the growth of human tumor xenografts in mice without causing any toxic effects in normal tissues and activates p53 selectively in tumor cells, whereas the effect in non-transformed cells is almost negligible (Issaeva et al., 2004). Interestingly, RITA affects p53's interaction with other proteins as well, such as p300, which binds N-terminus, C-terminal binding Parc, and iASPP which interacts with the Pro-rich region (Issaeva et al., 2004). These data indicate that the conformational change induced by RITA affects the whole p53 molecule (Issaeva et al., 2004).

The strong anti-proliferative and antitumor activity of NCS652287 has been previously reported and attributed to a DNA crosslinking effect that depends on the differential ability of tumor cell lines to take up and/or metabolize the compound (Nieves-Neira et al., 1999; Rivera et al., 1999). Although we cannot rule out the possibility that RITA metabolites can produce additional effects, our results obtained *in vitro*, in cells and *in vivo* suggest that the growth suppressor effect of RITA is mainly due to its ability to target p53 (Enge et al., 2009; Grinkevich et al., 2009; Hedstrom et al., 2009; Issaeva et al., 2004).

Recently reported DNA damage response induced by RITA resulting in phosphorylation of p53 and γ H2AX is also p53-dependent and can be detected only in wtp53-containing tumor cells (Yang et al., 2009b). Moreover, we identified an additional cellular target of RITA, namely the redox protein TrxR1. We demonstrated that RITA inhibits TrxR activity in cells in a p53-dependent manner, which might contribute to the tumor-specific killing properties of RITA (Hedstrom et al., 2009).

1.6.2 Different mechanisms evoked by Nutlin3a and RITA

Despite the fact that Nutlin3a and RITA target the same protein/protein complex, they induce different biological outcomes in tumor cells. Nutlin3a induces p53-dependent cell cycle arrest in cell lines in which RITA induces apoptosis and vice versa (Enge et al., 2009; Rinaldo et al., 2009). Understanding the mechanisms mediating these differences between RITA and Nutlin3a might facilitate the development of personalized medicine.

As mentioned above, RITA affects several p53-protein interactions. Similarly, the binding of Nutlin3a to Mdm2 promotes the displacement of several Mdm2 partner proteins, such as E2F, p21, hnRNPK and HIPK2 (Ambrosini et al., 2007; Enge et al., 2009; Rinaldo et al., 2009). At

the same time, disruption of the interaction between p53 and other proteins, for example MdmX, by Nutlin3a has not been reported (Bernal et al., 2010).

Thus, it appears that the disruption of p53/Mdm2 complex is not the only effect of these compounds: the binding of Nutlin3a changes the repertoire of Mdm2-binding partners, whereas RITA changes the p53 interactome. We think that these differences might result in the initiation of differential responses induced by p53 upon its release from Mdm2 by RITA or Nutlin3a. Therefore, Nutlin3a and RITA can serve as an excellent research tools to address p53 biology, and in particular, the mechanisms governing the p53 choice between triggering transcriptional programs leading to apoptosis or growth arrest.

Mdm2 plays an important role in life-death decision upon p53 reactivation

Rapid release of Mdm2 from p53 upon Nutlin3a or RITA treatment leads to a pool of free Mdm2 in cells. Since Nutlin3a binds MDM2, it can change the MDM2 interactome. Thus, it is possible that released Mdm2 will affect its other substrates differently upon Nutlin3a and RITA treatment. Does this have an impact on the biological response induced by these two compounds?

We have found that Mdm2 released from p53 upon RITA treatment promotes degradation of p21, the major p53 target responsible for cell cycle arrest. It also induces degradation of hnRNPK, the p53 cofactor required for the induction of p21 and of other cell cycle arrest genes. As a result, in the absence of robust p21 induction the p53-induced response is turned towards apoptosis. In contrast, Mdm2 bound by Nutlin3a does not degrade hnRNPK or p21, leading to a strong p21 up-regulation and induction of growth arrest (Enge et al., 2009).

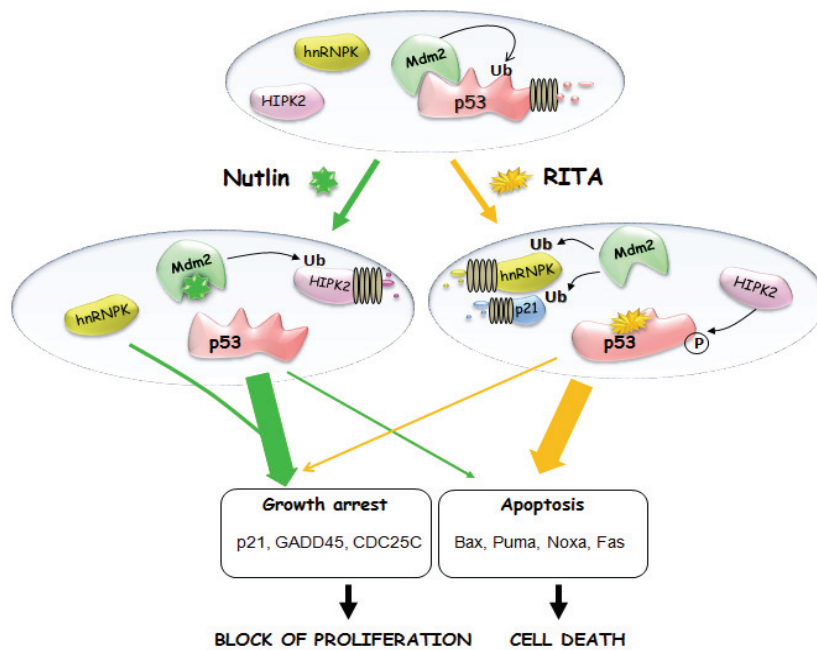


Figure 5. Different mechanisms evoked by Nutlin3a and RITA.

Several lines of evidence that inhibition of Mdm2 by small molecules differently affects Mdm2 interaction with its other substrates besides p53, comes from studies which showed that Nutlin3a disrupts interaction between Mdm2 and its partner proteins E2F1 and p73. Both factors interact with the N-terminal domain of Mdm2, where Nutlin3a binding site is located. As a result, in some p53-defective tumor cells (both p53 negative and p53 mutant), Nutlin3a in combination with chemotherapeutic drugs induces the expression of p53 targets Puma and p21 through the activation of E2F1 and p73 (Ambrosini et al., 2007, Kitagawa et al., 2008, Lau et al., 2008, Pierce and Findley 2009, Wang J., 2011, Ray 2011). Nutlin3a may therefore provide a therapeutic benefit in some tumors with mutant p53 when combined with chemotherapy (Ambrosini et al., 2007).

Another group has discovered that Mdm2 released by Nutlin3a targets the HIPK2 kinase for proteasomal degradation (Rinaldo et al., 2009). HIPK2 phosphorylates p53 at Ser46 which is required for the induction of pro-apoptotic genes by p53 (D'Orazi et al., 2002). In contrast, upon RITA treatment the level of HIPK2 and Ser46 phosphorylation of p53 are induced which leads to a potent induction of apoptosis in tumor cells (Rinaldo et al., 2009).

In addition, Mdm2 might have different activities upon its release from p53 by RITA and Nutlin3a due to posttranslational modifications induced by p53-dependent DNA damage signaling upon RITA treatment (Yang et al., 2009a). The mechanism of p53-mediated DNA damage response upon RITA treatment has not been elucidated yet and is currently under investigation.

In summary, a number of studies suggest that the binding of Mdm2 to its other partner proteins such as hnRNPk, p21 or HIPK2, can serve as a key switch regulating cell fate decision upon p53 reactivation (Figure 5). Since induction of cell cycle arrest might confer survival for some cancer cells, opening a possibility for tumor recurrence, we must understand the mechanism of this decision if we are to clinically exploit drugs activating p53.

1.6.3 Drug resistance and wtp53 reactivating compounds.

One of the main causes of failure of anti-cancer treatments is the development of *de novo* drug resistance by cancer cells. This is a very serious problem that leads to a disease recurrence or even death. Tumors can become resistant not only to the drugs used initially, but also to those to which they have not yet been exposed. *De novo* resistance is one of the major problems in drug development and anticancer treatments. Multiple mechanisms contribute to drug resistance, for example, loss of a cell surface receptor or transporter for a drug, changes in drug metabolism, mutation in the drug target or other mutations (Gottesman, 2002). Treatment with Mdm2 binding compounds creates a selection pressure for p53 mutations as a mechanism of *de novo* resistance.

In addition, recent study in AML samples from over 100 patients showed that a primary resistance to Mdm2 inhibitors exists despite the presence of wild-type *TP53* (Long et al., 2010). Mechanisms of resistance seem to be due to the insufficient p53 induction, defective p53 protein or compromised p53 - dependent apoptosis. These observations complicate the transition of such inhibitors into the clinic.

Clearly, translating the use of Nutlin3a and other p53 reactivating compounds to the clinic will be very much promoted by identifying molecular pathways that modulate the cellular response to treatment. To find cellular components that mediate drug cytotoxicity, shRNA

screens can be performed. shRNA screens also expose nodes whose inactivation can lead to further resistance to the drug. Brummelkamp et al. performed shRNA barcode screen to gain insight into the mechanism of Nutlin3a action (Brummelkamp et al., 2006). Inhibition of 14 genes resulted in the resistance of MCF7 cells to Nutlin3a. *TP53* was a top score, as well as p53 regulators 53BP1 and hnRNP K. Several p53 transcription targets were found in this shRNA screen, such as Bcl2 antagonist BAD, PLAB and IER3 (also called IEX-1). Other targets were found that have not been previously implicated in the regulation of the p53 pathway, such as TIEG (TGF β pathway), MYCL1, LHX3 (LIM homeobox protein 3), KCNK9 (potassium channel subfamily), RAB2 (Ras oncogenes family), GLRA2 (Glycine receptor isoform), and USP28 (ubiquitin specific protease) (Brummelkamp et al., 2006).

1.7 MOLECULES SIMULTANEOUSLY TARGETING WT AND MUTANT P53

Although mutations in the *TP53* gene target different residues, they ultimately affect the binding of p53 to DNA. The conformation of the wild type p53 DNA binding domain (DBD) is highly flexible (reviewed in Selivanova and Wiman, 2007). Most p53 mutations further destabilize this flexibility, resulting in p53 proteins with impaired sequence-specific DNA binding. As a result, mutant p53 proteins fail to activate transcription of p53 target genes and, hence, do not trigger a p53-dependent biological response. However, the functional activity of mutant proteins, even those most severely affected, such as His175, could be at least partially rescued at low temperature, suggesting that the defect is reversible (reviewed in Selivanova and Wiman, 2007). This leads to the idea that the binding of a ligand(s) that can stabilize the DBD will shift the balance towards active, properly folded form and thus restore the function to different mutant p53 proteins. Several small molecules reactivating mutant p53 have been identified, including CP-31398 (Foster et al., 1999), PRIMA-1 (Bykov et al., 2002), MIRA-1 (Bykov et al., 2005), STIMA-1 (Zache et al., 2008), p53R3 (Weinmann et al., 2008) and RETRA (Kravchenko et al., 2008), as well as rationally designed PhiKan083 (Boeckler et al., 2008), CDB3 (Issaeva et al., 2003) and SCH529074 (Demma et al., 2010). The mechanisms of action of these molecules are far from being clear, although most of them have been shown to inhibit the growth of human tumor cells carrying mutant p53 *in vitro*. Furthermore, CP-31398 and PRIMA-1 suppress the growth of human tumor xenografts in mice (Brown et al., 2009).

Mdm2 binding compounds, Nutlin3a and MI-219 have been shown to be efficient only in wtp53, but not in mutant p53- carrying tumors. A large cohort study of more than 100 primary CLL samples found direct correlation between wild-type *TP53* status and Nutlin3a- and MI-219-induced cytotoxicity (Saddler et al., 2008). However, despite inability of Nutlin3a to restore the function to mutant p53, it can induce growth suppression in some p53-null or mutant p53-carrying tumors through activation of E2F and p73 upon combination with cytotoxic drugs (Cheok et al., 2010; Kitagawa et al., 2008; Wang et al., 2011). Interestingly, several compounds which have been shown to restore the function of mutant p53 by interaction with its DBD, such as a synthetic peptide CDB3 and small molecules CP-31398 and SCH529074 (Demma et al., 2010) also reactivate wild type p53 by interfering with Mdm2-, but not HPV-E6-mediated degradation.

It is tempting to speculate that the binding of these molecules to the DBD induces a conformational change which prevents the docking of the central domain of Mdm2. Since efficient ubiquitination of p53 by Mdm2 requires both the N-terminus and DBD of p53 to be

involved in the interaction with Mdm2, preventing DBD/central domain interaction is sufficient to block, at least partially, the inhibitory effects of Mdm2 (Demma et al, 2010). The mechanism of activation of wild type p53 by two other mutant p53-reactivating molecules, PRIMA-1Met (Apr-246) (Bykov et al., 2002) and p53R3 (Weinmann et al., 2008), are currently unclear. p53R3 enhances the recruitment of both wt and mutant p53 to target promoters and induces the expression of a number of p53 target genes (Weinmann et al., 2008). However the effects on wtp53 stability and/or binding to Mdm2 have not been studied yet.

Our lab has recently found that PRIMA-1^{MET}/Apr-246 activates p53 transcriptional activity and induces p53-dependent apoptosis in wt p53 carrying melanoma cells *in vitro* and *in vivo* without affecting p53 level or stability (Bao et al., 2011). In addition, PRIMA-1^{MET}/Apr-246 induces apoptosis in AML patient samples irrespectively of p53 status (Nahi et al., 2008). However, PRIMA-1^{MET}/Apr-246 does not affect either Mdm2 or E6-mediated ubiquitination (Demma et al., 2010). It is possible that alkylation of p53 by PRIMA-1^{MET}/Apr-246 (Lambert et al., 2009) may play a role, but the exact mechanism awaits further investigation.

Several groups have reported that the N-terminal domain of p53 can affect the folding of the core (reviewed in Selivanova and Wiman, 2007). Perhaps the most striking observation has been reported by D. Lane and coworkers, who showed that the deletion of four N-terminal residues restored mutant p53's conformation and transcriptional activity (Liu et al., 2001). It is thus tempting to speculate that some N-terminal binding molecules might be able to restore the wt p53 conformation to mutant p53.

In conclusion, at least several compounds which directly target p53 might be applicable for the treatment of a wider range of tumors, irrespectively of mutant or wild type p53 status. More detailed elucidation of the structural features of the full length p53 will be helpful in order to better understand the mechanism of action of p53-reactivating compounds. Although p53 has proven to be a challenge for structural biologists due to its high conformational flexibility, the first steps in this direction have been undertaken (Okorokov et al., 2006; Tidow et al., 2007).

1.8 DRUG COMBINATIONS FOR ANTICANCER TREATMENT

To avoid drug resistance, cocktails of drugs with different mechanisms of entry into cells and different cellular targets could be used to allow for effective chemotherapy and high cure rates. Drug combinations can improve single therapeutic agents by being more effective via targeting heterogeneous populations of malignant cells. Mdm2 inhibitor Nutlin3a is a very good example on how drug combinations might work. Nutlin3a has been shown to synergize with a wide variety of cytotoxic agents including those targeting tubulin (vinblastine), cyclin-dependent kinase inhibitors (roscovitine), aurora kinase inhibitors (vX-6807), DNA-damaging agents (doxorubicin), S-phase agents (fludarabine and gem citabine), and radiation (reviewed in Cheok et al., 2011). In addition, Nutlin3a synergizes with tumor necrosis factor α -related apoptosis-inducing ligand (TRAIL) in AML (reviewed in Cheok et al., 2011). Moreover, cyclotherapy, i.e., selective killing of mutant p53 cells by anti S-phase or antimetabolic drugs in combination with the reversible induction of growth arrest in normal tissues by the p53-reactivating compound is another way to use drug combinations (Lane et al., 2010). Published data provides a rationale for drug combinations to minimize side effects. Further, application of drug combinations targeting different pathways is expected to reduce the chance of acquired resistance in patients.

2 AIMS AND RESULTS

The thesis is based on three papers exploring molecular mechanisms of reactivation of the p53 tumor suppressor function by the small molecule RITA.

2.1 PAPER 1

ABLATION OF KEY ONCOGENIC PATHWAYS BY RITA-REACTIVATED p53 IS REQUIRED FOR EFFICIENT APOPTOSIS OF TUMOR CELLS

V. V. Grinkevich, F. Nikulenkov, Y. Shi, M. Enge, W. Bao, A. Maljukova, O. Sangfelt, A. Gluch, A. Kel, and G. Selivanova. *Cancer Cell* 15 (2009) May 5, 441-453.

The aim of this study is to answer several important questions, how RITA inhibits growth of tumor cells of different origins; which signaling pathways are involved in the p53-mediated response; and to get insight into basic tumor-suppressing mechanisms by p53.

RESULTS

By means of microarray analysis and further validation by PCRs and immunoblotting we identified a set of oncogenes repressed by RITA in colon carcinoma HCT116 and breast carcinoma MCF7 lines. These include *IGF1R*, *PIK3CA*, *PIK3CB*, *MYC*, *MCL1*, *BCL2*, *MAP4* and *BIRC5* (survivin). We confirmed the data obtained *in vitro* using mouse xenografts *in vivo*. Observed effects were p53-dependent, since we could not detect the inhibition of these factors in p53-null cancer cells (HCT116 TP53^{-/-}, Saos2 and H1299). Additionally, pretreatment of cells with p53 inhibitor PFT α abolished the effect of RITA.

We found that RITA-reactivated p53 induced apoptosis and downregulated survival factors only in cancer cells, but not in nontransformed MCF10A or HDF's, in contrast to known DNA-damaging compound 5FU.

Transcriptional repression of the members of the IGFR-PI3K signaling pathway by RITA-reactivated p53 resulted in the inhibition of Akt-kinase activity, inhibition of Akt target kinase mTOR and activation of Akt target GSK3 β . Inhibition of mTOR and transcriptional downregulation of another translational factor eIF4E lead to the downregulation of CAP-dependent and CAP-independent translation. At the same time, activation of GSK3 β promoted degradation of its targets c-Myc, Mcl-1 and β -catenin.

We found that restoration of p53 can block survival and pro-proliferative factors, such as c-Myc, at three different levels: by inhibiting their transcription, translation and inducing their proteasomal degradation. Proteasomal degradation of oncogenes c-Myc and cyclin E was due to the enhanced expression of FBXW7 (hCdc4).

Unexpectedly, we found that the response of tumor cells to different doses of RITA (0.1 and 1 μ M) was similar in terms of induction of p53 and its apoptotic targets, but oncogenes were regulated differently. 1 μ M RITA was sufficient to trigger downregulation of oncogenes while upon 0.1 μ M RITA the decline was either absent or less pronounced. Notably, only 1 μ M RITA induced apoptosis in tumor cells, indicating that inhibition of oncogenes contributes to apoptosis induction by p53.

To address the mechanisms underlying the differential regulation of pro-apoptotic and pro-survival genes by p53 we examined the sub-cellular distribution of p53 upon treatment with 0.1 and 1 μ M RITA. We found that chromatin-bound p53 fraction increased, while Mdm2 in chromatin fraction decreased in a dose-dependent manner. However, both concentrations of RITA reduced the amount of p53/Mdm2 complexes and induced p53 accumulation in a soluble fraction to the same extent.

Further, we compared the relative abundance of p53 and Mdm2 on p53-activated versus p53-repressed promoters using chromatin immunoprecipitation (ChIP). We found that in untreated cells the p53/Mdm2 ratio on p53-activated *CDKN1A* promoter was significantly higher, than on p53-repressed *MCL-1* promoter. Treatment with 0.1 μ M RITA increased p53/Mdm2 ratio only on *CDKN1A* promoter, whereas 1 μ M RITA increased p53/Mdm2 ratio on both promoters. It is therefore possible that transactivation of p53 might be less tightly controlled by Mdm2, than transrepression. Analysis of microarray data revealed that a number of genes activated by p53 had higher basal expression level in p53-positive, than in p53-null cells. On the contrary, the mRNA levels of p53-repressed genes did not differ between the cell lines. Therefore, the dose-dependent effect of RITA on the expression of oncogenes appears to be due to a less efficient release of Mdm2 from the promoters of p53-repressed genes.

To address the question of whether inhibition of oncogenes is essential for the induction of apoptosis by p53, we applied genetic and pharmacological approaches. We selected three representative oncogenes - pro-survival and pro-proliferation factor Akt, pro-proliferative c-Myc, and anti-apoptotic Mcl-1. We found that the combination of low dose of RITA or Nutlin3a with the inhibition of oncogenes by a chemical inhibitor or corresponding siRNA resulted in a synergy in apoptosis induction.

Our study adds another dimension to the p53 story, demonstrating that p53 reactivation triggers the ablation of crucial oncogenes. The multitude of oncogenes inhibited by p53 and the multiple levels on which they are targeted create external robustness of the p53 response. This capability might allow p53 to cope with the daunting challenge of anticancer therapy: multiple genetic abnormalities in individual cancers. We show that the threshold for p53-mediated transrepression of survival genes is higher than for transactivation of proapoptotic targets. Inhibition of oncogenes by p53 reduces the cell's ability to buffer proapoptotic signals and elicits robust apoptosis. Our study highlights the role of transcriptional repression for p53-mediated tumor suppression.

2.2 PAPER 2

TARGETING p53 RESIDUES S33 AND S37 BY RITA INHIBITS P53/MDM2 AND p53/MDMX INTERACTIONS VIA AN ALLOSTERIC MECHANISM

J. Zawacka-Pankau, V. V. Grinkevich, A. Vema, K. Ridderstråle, E. Hedström, C. Spinnler, V. Andreotti, A. Inga, L-G. Larsson, A. Pramanik, A. Karlén, A. L. Okorokov, and G. Selivanova. **Manuscript**

The aim of this paper was to elucidate the ability of RITA to bind p53 in cellular context and to elucidate the mode of RITA action by mapping the binding site of RITA in the p53 N-terminus.

RESULTS

To test whether RITA targets p53 in a cellular context, we treated HCT116 cells carrying wild-type p53 and HCT116 p53-null cells with [¹⁴C]-RITA. We separated RITA/protein complexes using SDS-polyacrylamide electrophoresis and compared the spectrum of RITA-

bound proteins between these isogenic cell lines. RITA/protein complexes were evident in p53-positive, but not in p53-null cells. Position of the major radiolabeled band coincided with that of p53, which was further confirmed by p53 immunodepletion.

RITA/protein complexes were rather unstable and were easily disrupted upon conventional SDS-electrophoresis. Therefore we developed a small-molecule band shift assay using native gel electrophoresis. We confirmed that [14C]-RITA/protein complex in HCT116 cells coincided with that of p53 and was absent in p53-null cells.

We found that [14C]-RITA binds to Glutathione-S-transferase (GST)-fusion N-terminal p53 peptide (Np53) and to HSA (human serum albumin). HSA is known to bind diverse ligands and to serve as an important blood carrier of various molecules and therefore served as a positive control. RITA did not associate with GST protein itself nor with human fibrinogen, suggesting the selective interaction of RITA with p53. This is in line with our previous FCS results showing that RITA selectively interacts with the recombinant N-terminus of p53, but not with GST, EBNA2, or Mdm2 proteins (Issaeva et al., 2004).

In order to identify p53 residues involved in the interaction with RITA, we generated a series of the N-terminus deletion mutants (2-25N, 10-65N, 20-65N, 38-58N, 25-65N and 35-65N), tested their interaction with RITA and found that RITA-binding site is located between residues 25 and 38 in the proximity to L35.

Based on the available information on the structural organization of the p53 N-terminus and our deletion analysis data, we carried out a molecular modeling of RITA/p53 complex by using Monte Carlo conformational Search. The resulting model implies that the binding of RITA involves the formation of hydrogen bonds between its terminal hydroxyl groups and S33 and S37 in 33-SPLPS-37 sequence of p53, as well as hydrophobic interactions with the residues P34 and P36 via one of the thiophene and the furan rings of RITA.

Our model suggests that the combination of hydrogen bonds and hydrophobic interactions between RITA and the SPLPS sequence increases the rigidity of the prolin-containing SPLPS motif. This produces a conformational trap which limits the flexibility of the amphiphatic α -helix of the N-terminus and constraints the process required to expose F19, W23 and L26 residues interacting with Mdm2.

The prediction from our model is that the conformational change induced by RITA will impinge not only on Mdm2, but also on other proteins interacting with the N-terminus of p53, for example, Mdm2 homolog MdmX. By using two-site ELISA *in vitro* and co-immunoprecipitation experiments *in vivo* we found that, indeed, RITA efficiently inhibited the binding of MdmX to p53. MdmX, as well as Mdm2, requires the formation of the α -helix in p53 and exposure of the same residues F19, W23 and L26. Thus, RITA differs from previously described Mdm2 inhibitors, which do not disrupt the p53/MdmX interaction.

Molecular modeling showed the absence of efficient interaction between p53 and RITA analogs lacking one or two terminal hydroxyl groups, NSC-672170 and NSC-650973-N respectively. By using FCS and competition assays we confirmed that RITA analog lacking both hydroxyl groups (NSC-650973-N) does not bind to p53. Further, by using *in situ* proximity ligation assay, which allows visualizing protein-protein interactions in cells, we found that this analog does not prevent p53/Mdm2 interaction. As a result, NSC-650973-N did not induce p53 accumulation and did not suppress the growth of tumor cells. Another analog, NSC-672170 lacking only one hydroxyl group, suppressed the growth of HCT116 cells with IC50 2 μ M, but still less potent than RITA (IC50 0.05 μ M). Thus, our experiments suggest

that both terminal hydroxyl groups of RITA are required for the efficient binding to p53 and that RITA's ability to bind p53 correlated with the prevention of p53/Mdm2 binding, induction of p53 and induction of apoptosis.

Further validation of our model addressed the role of S33 and S37, predicted to be critical for RITA/p53 interaction. Indeed, we found that the interaction of [¹⁴C]-RITA with mutant GST-N terminal peptides Np53(S33A) and Np53(S33A/S37A) was reduced in comparison to wild type Np53 protein.

Our previous results demonstrated the absence of growth suppression by RITA in mouse tumor cell lines (Issaeva et al., 2004 and data not shown). We found that mouse p53 lacks residues corresponding to S33 and P34 of human p53 (NCBI-BLAST) and that binding between [¹⁴C]-RITA and mouse p53 was not efficient. In line with these results, we could not detect the disruption of p53/Mdm2 interaction and p53 accumulation upon RITA treatment in mouse tumor cells. Notably, the expression of human p53 in mouse embryo fibroblasts, derived from transgenic mice expressing only human p53 (SWAP MEF), transformed with c-Myc or Ras oncogenes, restored the ability of RITA to induce p53 and to kill mouse transformed cells.

By using yeast-based functional assay which measures p53 transcriptional activity we found that co-transfection of Mdm2 with wtp53 or S33/S37p53 inhibited transcriptional activity of both proteins. Treatment with Nutlin3a relieved the activity of both p53 proteins. In contrast, RITA treatment relieved only wtp53-mediated transactivation, but not the S33/S37 mutant.

Finally, we generated RKO and SW48 colon carcinoma cell lines stably expressing wtp53 or S33A/S37A mutant. We found that Nutlin3a induced accumulation of wt and S33A/S37A p53 with similar efficiency. In contrast, RITA was able to induce accumulation of wtp53, but the induction of the serine mutant was impaired. Thus, mutations S33 and S37 prevent the activation of p53 by RITA.

Conclusions:

Our findings implicate the conformational state of the 33-SPLPS-37 (S33 and S37 in particular) sequence distal from the Mdm2-interacting residues as a key structural element regulating p53/Mdm2 interaction. This regulatory mechanism could be modulated by small molecules such as RITA.

2.3 PAPER 3

RESCUE OF THE APOPTOTIC- INDUCING FUNCTION OF MUTANT P53 BY SMALL MOLECULE RITA

C. Y. Zhao, V. V. Grinkevich, F. Nikulenkov, W. Bao and G. Selivanova. *Cell Cycle*. (2010) May;9(9):1847-55.
The aim of this paper was to investigate whether RITA can reactivate mutant p53.

RESULTS

Around half of all human tumors carry point mutations in the p53 gene that abolish p53 DNA binding. Tumors that carry mutant p53 often show increased resistance to conventional chemo- and radiotherapy which correlates with poor prognosis. Thus, reactivation of mutant p53 appears to be especially important. Since RITA binds the N-terminus of p53 and induces a conformational change which prevents the binding of several proteins to both N- and C-

terminus of p53 (Issaeva et al., 2004) we decided to test whether that RITA might work on mutant p53 as well.

Here, we found that RITA suppressed the growth and induced apoptosis in a p53-dependent manner in a variety of cell lines that harbor different p53 mutants. We tested a number of hot spot mutants including His175, His273 and Trp248; as well as a set of Burkitt's Lymphoma cell lines that carry Gln248, Arg213, Thr234, Arg283 and Ile254 mutants. The cell death induced by RITA is caspase-dependent and resulted in classic hallmarks of apoptosis such as PARP cleavage, cytochrome c release and appearance of apoptotic bodies.

To find out whether observed effect on mutant p53-containing cells is p53-dependent we inhibited p53 by Pifithrin- α or depleted by shRNA, and also used p53-null cells. Inhibition of p53 by different means antagonized RITA-induced cell deaths.

The kinetics of apoptosis induction by RITA greatly varied in different cell lines (from 6 hours to 4 days). This did not depend on a type of mutation, since His 273 mutant cell lines A431, SW480 and HT29 undergo apoptosis within 6, 24 and 48 hours, respectively. Observed effect did not depend on the type of the tissue as well. The cause of the time variation remains unclear, although we speculate that efficiency of drug uptake/efflux might contribute.

We found that RITA treatment of mutant p53 tumor cell lines induced transcription of known p53 targets p21, BAX, PUMA and GADD45, suggesting that the mutant p53 restored by RITA is transcriptionally functional. Further, mRNA and protein levels of oncogenic factors Bcl-2, c-Myc, Mcl-1 and IGF-1R were downregulated upon treatment with RITA.

Thus, RITA is a promising lead for the development of anti-cancer drugs that reactivate the tumor suppressor function of p53 in cancer cells irrespective whether they express mutant or wild type p53. This might contribute to the efficiency of the treatment and reduce the chance of *de novo* resistance.

3 ACKNOWLEDGMENTS

It has been a fantastic six-year chapter of my life and I would like to express my gratitude to the people who have helped me during these years.

Galina Selivanova, my supervisor and mentor: for giving me opportunity to do a PhD even though you were looking for a postdoc, for introducing me to the p53 field and to the members of global p53 community. For giving me exciting projects and for always finding time to discuss them. Dear Galina, thank you very much for being so kind to me, patient and inspiring! You taught me, that in order to achieve something big, you need to aim high.

Joanna Zawacka-Pankau, my co-supervisor: for sharing your knowledge and experience. It was a pleasure to work with you; your energy and enthusiasm always encourage me.

Natalia Issaeva, my first friend in Sweden: For all your support in science and in life. I am so happy that I met you and your wonderful family!

All the present and past members of Selivanova group who have helped me in one way or another and for all the good times (barbecues, parties, discussions and lunches): Fedor, Yao, Andreas, Clemens, Misha and Hai. Ying, Elizabeth and Martin (for introducing me to the Swedish culture). Diana, Sabine and Wenjie (for your kindness and support). Thank you, for creating a great atmosphere in our lab!

Special thanks for all coauthors of the papers included in this thesis. Thank you for all your contribution and hard work.

My fantastic collaborators: Andrej Gudkov and his group, Katerina Gurova, Katja Pokrovskaja, Lars-Gunnar Larsson and his group, Olle Sangfelt and Andrej Okorokov. Thank you for the support, interesting discussions and help with projects.

All members of MTC, for creating a unique atmosphere of sharing ideas, reagents and for being so helpful. Particularly Marie Henriksson and her group, Sonia Lain and her group, Mikael Rhen and his group, Martin Rottenberg and his group, Anna Lögdberg, Anita Wallentin and Helene Stambeck. My friend Lesja, for always having time for a cup of tea and for giving helpful advises.

My friends Masha from Lyon, Masha from Boston, Sveta and her family from St. Petersburg, Sveta from Ismail, Sasha, Oika, Genja, Lesha, Kostja, Lena, Nicolas and Emilie (French spark in my life), my god mother Vera, t. Tanja, , t. Natasha and Tatjana I. Merkulova. Thank you all for the friendship and support!

All teachers that contributed to my development: especially teacher of physics Vladimir Shelest, Valery Pospelov and his group, Masha Abramova and Anna Pendina.

My family from Russia, Ukraine and Romania.

My mother. For all your love! For everything that you did for me. For being an example of a great scientist and for your enthusiasm!

My beloved, husband and colleague Aurel. Thank you for your love and for our small angel Maria! Thank you for your support during thesis preparation!

My daughter Maria, you completely changed my way of looking at life. Your smile helps me forget all the problems. Thank you for giving me a chance to rediscover the world.

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