p53-Based cyclotherapy: exploiting the 'guardian of the genome' to protect normal cells from cytotoxic therapy

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Side effects of chemotherapy are a major impediment in the treatment of cancer. Cyclotherapy is an emerging therapeutic strategy for protecting normal cells from the side effects of chemotherapy. Low, non-genotoxic doses of known p53 activators can be used to induce p53-dependent cell cycle arrest in normal cells bearing wild-type p53. This cytostatic effect of p53 can protect normal cells from the toxicity of S- or M-phase poisons. Here, we have reviewed existing cyclotherapy regimens using two well-known p53 activators, nutlin-3 and actinomycin D. We have highlighted an exemplar clinical perspective for cyclotherapy in breast cancer. The recent development of novel stapled peptides as activators of p53 without the corresponding cytotoxicity holds great promise for cyclotherapy to enhance the therapeutic window of existing chemotherapy drugs.

Current treatments for cancer comprise surgery, radiotherapy, chemotherapy, endocrine therapy and targeted therapies. Modern chemotherapy is a systemic approach to treat cancer compared with the locoregional approaches of surgery and radiation therapy, and uses chemical agents (anticancer or cytotoxic drugs) to interact with cancer cells and eradicate or control the growth of cancer.

The purpose of the chemotherapy is to selectively kill tumour cells. Approximately 50 different anticancer drugs are available for clinical practice and an equal number are currently being tested in clinical trials. The most desirable drug will be one that destroys the cancer cells, leaves normal cells unharmed, and causes the fewest unpleasant and undesirable side effects. The therapeutic goal is to favourably balance the risk–benefit ratio, in which the morbidity and toxicity of chemotherapeutic agents are weighed against the potential benefits. Classic chemotherapeutic agents preferentially target rapidly dividing cells, whether cancer cells or normal cells. Therefore, selectivity towards cancer cells by standard chemotherapeutic drugs is limited and toxicity to normal tissues remains a therapeutically limiting problem in the clinic.

Suppression of bone marrow activity, which results in a decrease in white cell, platelet and red blood cell production, is a key limiting factor in standard chemotherapy. Because chemotherapy is most effective when used at the highest tolerated dose, the interval between treatments may need to be prolonged to prevent life-threatening bone marrow suppression. Supportive measures such as prophylaxis for or treatment of bone marrow suppression include granulocyte-stimulating factors and blood product transfusion. Other undesired consequences of standard chemotherapy include hair loss, mucositis, gastrointestinal tract toxicity, dermatological toxicity, cardiotoxicity and asthenia. In addition, most classic chemotherapeutics are highly mutagenic either by causing damage to DNA (directly or indirectly) or, as in the case of mitotic poisons, by disrupting chromosome distribution. This can lead to an increased risk of second tumours later in life for those who survive the cancer.

P53-BASED CYCLOTHERAPY

Given the clinical problem of differential chemotherapeutic effects on tumour vs normal cells, cyclotherapy is a promising strategy to protect normal tissues from the side effects of chemotherapy (Blagosklonny and Pardee, 2001). Here, we briefly review key facets of the p53 gene and focus on p53-based cyclotherapy. This approach exploits the key genetic difference of the p53 status between a normal cell and a p53 mutant bearing cancer cell and therefore is relevant to patients suffering from cancers carrying inactivating mutations in the p53 gene.

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As shown in Figure 1, *p53* activation by low doses of *p53* small-molecule activators can be used to selectively guard normal cells from the killing effects and genomic instability induced by two specific types of standard chemotherapeutics (S-phase and mitotic poisons). Small-molecule *p53* activators have no effect on *p53* mutant bearing cancer cells, so these cells remain susceptible to the S phase or M phase targeting chemotherapeutic drug.

Activation of wild-type *p53* in normal tissues may cause p53-related toxicities. For *p53*-based cyclotherapy, only low doses of *p53* activators are used to specifically achieve the 'cytostatic' rather than the 'cytotoxic' effects of *p53* activation in normal tissues.

THE REVERSIBLE CYTOSTATIC EFFECTS OF P53

The *p53* gene, also known as 'The Guardian of the Genome' (Lane, 1992), is located on the short arm of chromosome 17 (17p13.1) (Isobe *et al*, 1986). The inheritance of only one functional copy of the *p53* gene causes a familial syndrome called Li-Fraumeni syndrome and these patients are predisposed to cancer (Malkin *et al*, 1990). Studies on *p53*-null mice show that although viable (and seemingly normal), they are prone to early tumour development (Donehower *et al*, 1996). A major biological function of *p53* is to respond to stress signals and activate the transcription of downstream target genes involved in important cellular mechanisms like cell cycle control, DNA repair and apoptosis. For the cell cycle control mechanisms, *p53* has two very distinct roles. The first is a protective (cytostatic) one in which p53 arrests cells in the G1 phase of the cell cycle upon sensing DNA damage. p53 thereby prevents cells from multiplying damaged DNA via the production of p21, which interacts with a cell division-stimulating protein (cdk2). With p21 bound to cdk2, a cell cannot pass through to the next phase of the cell cycle (Figure 2). In the absence of functional *p53*, p21 protein is not increased and cannot act as the 'stop signal' for cell division. In a second role, p53 initiates apoptosis upon irreparable damage to the cell.

Various different cellular signals like stress due to DNA damage, activation of oncogenes, hypoxia and nutrient deprivation can induce p53 transcriptional activity. The specific response of *p53* to these different cellular stresses depends on post translational modifications like phosphorylation and acetylation. In addition, it also depends on p53 interaction with its partners such as Mdm2. p53 levels are tightly regulated by Mdm2, an E3 ubiquitin ligase that causes proteasomal degradation of p53 (Toledo and Wahl, 2006). Interestingly, p53 protein transcriptionally activates Mdm2 to form a negative feedback mechanism, which maintains low p53 levels under normal, unstressed conditions. During stress activation, for example DNA damage, ATM/ATR kinases phosphorylate both p53 and Mdm2 proteins causing disruption in the interaction between the two. This phosphorylation facilitates p53 protein stabilisation leading to the transactivation of p53 target genes (reviewed in Toledo and Wahl, 2006). During oncogene activation, induction of another tumour suppressor protein p14ARF (known as p19ARF in mice) can also cause p53 protein stabilisation as p14ARF has been directly shown to bind Mdm2 and prevent the p53 degradation (Weber *et al*, 1999).

DNA damage has been shown to cause *p53* transcriptional activity resulting in p21 induction that can result in both G1 and G2 arrest (Vogelstein *et al*, 2000). Even low levels of DNA damage can cause G2 arrest and non-entry into M phase in p53 wild-type HCT116 cells (Bunz *et al*, 1998). Inducible systems have demonstrated that p53 overexpression can result in both G1 and G2 arrest, and that prolonged overexpression of p53 could downregulate Cyclin B1 (Agarwal *et al*, 1995). As the Cyclin B1/Cdc2 complex is probably the major regulatory factor required for entry into mitosis, decreased expression of Cyclin B1 and inhibitory phosphorylation of Cdc2 are thought to be the major mediators of G2 arrest. Overexpression of p21 also causes cells to accumulate in G2 and is associated with a reduction of Cyclin B1-associated kinase activity (Niculescu *et al*, 1998). Expression of p21 may also be associated with a brief delay in G2, probably causing late cell cycle checkpoints in normal cycling cells (Dulic *et al*, 1998).
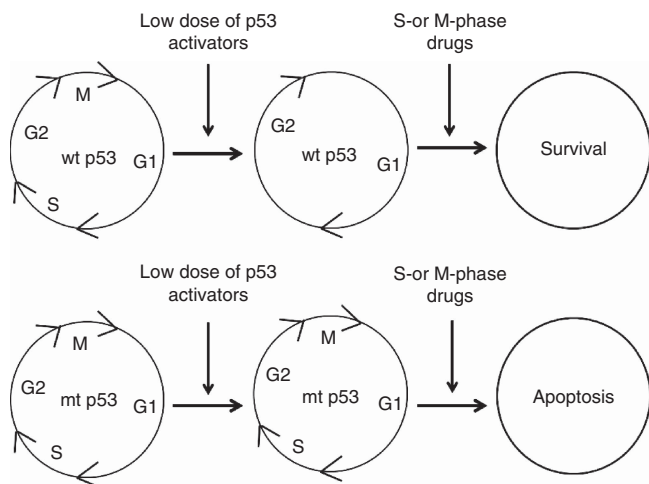

Figure 1. Principle of *p53*-based cyclotherapy: cyclotherapy, unlike conventional chemotherapy, distinguishes cancer cells from normal cells. Pretreatment with a *p53* activating drug halts the cell cycle in G1/G2 only in normal cells carrying wild-type *p53*, whereas *p53*-mutant cancer cells continue cycling into S and M phase. Adding an S-phase or M-phase poison should then specifically target *p53*-mutant cancer cells to bring about apoptosis, while having no effect on cell cycle-arrested normal cells.

P53-BASED CYCLOTHERAPY USING NUTLIN-3

p53-deficient or *p21*-deficient HCT116 colon cancer cells enter mitosis despite pretreatment with doxorubicin, a DNA-damaging drug. This pretreatment allowed for the protection of only the wild-type *p53* HCT116 cells from the cell death induced by taxol (paclitaxel) and not the *p53*- or *p21*-deficient HCT116 cells (Blagosklonny *et al*, 2000). However, studies have also shown that pretreating with doxorubicin can also induce growth arrest in some *p53*-deficient tumours, hence protecting them from taxol (Blagosklonny, 2002). This undesired effect of doxorubicin suggested that, more *p53*-selective compounds are required for cyclotherapy to be a successful approach.

Nutlin-3 has been the forefront candidate for p53-based cyclotherapy. Nutlins are a group of cis-imidazoline analogues, first identified by Vassilev *et al* (2004). These compounds have a high binding potency and selectivity for one of the p53 binding sites on Mdm2. Crystallisation data have shown that nutlin-3 mimics the three residues of the helical region of the transactivation domain of p53 (Phe19, Trp23 and Leu26) that are conserved across species and critical for binding to Mdm2. In this way, nutlin-3 prevents effective binding of p53 to Mdm2.

Two different groups demonstrated the protective role of nutlin-3 via the p53/Mdm2 mechanisms using the isogenic colon cancer cell lines HCT116 and HCT116*p53* − / − (Carvajal *et al*, 2005; Kranz and Dobbelstein, 2006). The first was a sequential combination of nutlin-3 and taxol, a well-known and widely used mitotic inhibitor. Pretreating wild-type *p53* cells with nutlin-3 before adding taxol caused these cells to arrest in G1 or G2 of the
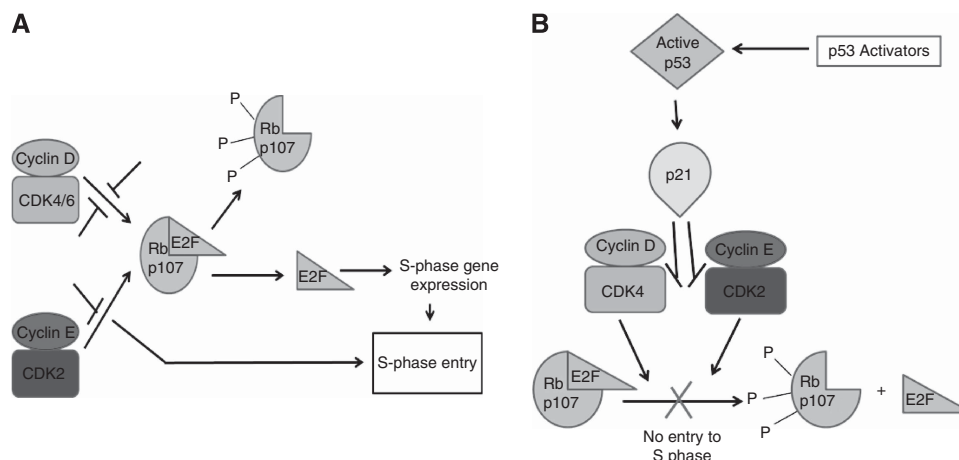
A

B

Figure 2. (**A**) Schematic to show the processes involved during S-phase entry. Rb phosphorylation is an important step during the regulation of normal cell entry to the S phase. (**B**) Upon addition of a low dose of *p53* activating drug, as a result of p53 transcriptional activity, the cdk inhibitor p21 is activated. p21 transcription results in Rb hypophosphorylation via CDK4 and CDK2 inhibition leading to growth arrest. Cells would then not gain entry into S phase.

cell cycle, hence protecting them from taxol-induced apoptosis. This arrest was due to *p53*-dependent activation of p21 only upon pretreatment with nutlin-3 or its active enantiomer nutlin-3a and only when cells encoded wild-type *p53*. In addition, sequential combination also protected proliferating skin fibroblasts from taxol with this arrest reversible upon drug removal (Carvajal *et al*, 2005).

Subsequently, a similar sequential combination was carried out and cells carrying functional *p53* were shown to be protected by nutlin-3 pretreatment from the cytotoxicity of the S-phase inhibitor gemcitabine. In contrast, isogenic cells lacking functional *p53* continued to enter S-phase regardless of nutlin-3 pretreatment and remained highly susceptible to gemcitabine-mediated cytotoxicity. The sequential treatment with nutlin-3 alone, followed by transient exposure to nutlin-3 plus gemcitabine, efficiently killed tumour cells with deletions or mutations of *p53* but largely spared the non-transformed human keratinocytes (Kranz and Dobbelstein, 2006). The positive results described in these two cell culture studies (Carvajal *et al*, 2005; Kranz and Dobbelstein, 2006) have been confirmed and extended by experiments using two breast cancer cell lines expressing mutant *p53* (van Leeuwen *et al*, 2012).

To date, there is only one report of a *p53*-based cyclotherapy study *in vivo*. In this study, nutlin-3 pretreatment was shown to protect mice from neutropaenia induced by the Polo-Like-Kinase-1 (PLK-1) inhibitor BI2536 (Sur *et al*, 2009). In addition, xenograft studies showed that pretreatment with nutlin-3 did not interfere with the anti-tumour effect of BI2536. This is a remarkable result and moves the potential use of nutlin-3 for the application of cyclotherapy closer to the clinic.

ACTINOMYCIN D, AN OLD CHEMOTHERAPY DRUG, FOR P53-BASED CYCLOTHERAPY

Actinomycin D (ActD), like nutlin-3, is a potent activator of *p53*. ActD was the first antibiotic shown to have anti-tumour activity. This old chemotherapy agent had been used in the clinic for the treatment of children with rhabdomyosarcoma, Wilms' tumour and Hodgkin's disease (Farber *et al*, 1960). Low, nanomolar, doses of ActD are not highly genotoxic (Foster *et al*, 1994), effectively increase p53 levels and transcription function and induce the expression of a panel of genes that overlaps with nutlin-3-induced genes (Choong *et al*, 2009). In addition, this highly

potent compound can cause *p53*-dependent reversible cell cycle arrest in normal keratinocytes (Choong *et al*, 2009).

Despite the apparent similarities between the effects of ActD and nutlin-3 in cells, the mechanisms of action are very different. Whereas nutlin-3 directly binds to Mdm2, ActD inhibits RNA synthesis by binding to GC-rich regions in DNA and is especially effective at disrupting ribosomal RNA biosynthesis (Goldberg, 1971). This causes a nucleolar stress that is sensed by p53 (Rubbi and Milner, 2003). According to the current model, nucleolar stress caused by ActD is able to enhance the interaction between Mdm2 and ribosomal proteins, such as L11, resulting in the impairment of Mdm2-dependent degradation of p53 (Sundqvist *et al*, 2009).

Encouraged by the remarkable results obtained by combining nutlin-3 with S- and M-phase poisons and the similar p53-dependent effects of low-dose ActD and nutlin-3, we have investigated ActD for application as cyclotherapy. We found that low, non-genotoxic, doses of ActD can induce a reversible cytostatic effect in normal proliferating fibroblasts and protect them from the aneuploidy induced by VX-680 (Rao *et al*, 2010). However, pretreating cells bearing mutant *p53* or lacking *p53* with this low dose of ActD also prevented the appearance of polyploidy in these cells. Even so, this pretreatment did not weaken the effects of VX-680 in clonogenic assays (Rao *et al*, 2010).

Subsequently, we investigated ActD in combination with the nucleoside inhibitor gemcitabine. Here, we found that pretreating with low, non-genotoxic doses of ActD, prior to adding gemcitabine, does not protect tumour cells bearing mutant *p53* or lacking *p53*. Pretreating normal proliferating fibroblasts, or wild-type *p53* tumour cells with low doses of ActD, indeed protected these from the toxic effects of gemcitabine (van Leeuwen *et al*, 2012).

Thus, ActD as a clinically approved drug is an exciting candidate for cyclotherapy. However, further studies using *in vivo* models need to be developed before this approach can reach clinical practice.

P53-BASED CYCLOTHERAPY FOR BREAST CANCER: A CLINICAL PERSPECTIVE

Breast cancer survival is increasing due to early detection and advances in treatment. However, there remains a need for more effective and better tolerated treatments. Chemotherapy is used as

adjuvant treatment for early-stage invasive breast cancer to prevent recurrence and is also used to treat advanced breast cancer.

Triple negative breast cancers (TNBCs) are defined as tumours that lack expression of oestrogen receptor (ER), progesterone receptor (PR) and HER2 receptor. Triple negative breast cancers are aggressive and therapeutically challenging breast cancers currently treated with a combination therapy involving surgery, radiotherapy and chemotherapy. Triple negative breast cancer account for 17% of all breast cancers. Prominent features of TNBC include overexpression of epidermal growth factor receptor (EGFR), a high proliferative rate, frequent genomic alterations, phenotypic similarity to BRCA1-associated cancers and frequent mutations of the *p53* gene with the corresponding protein heterogeneously expressed. The established chemotherapy for triple negative breast cancer includes combination chemotherapy including anthracyclines such as doxorubicin or epirubicin, taxanes such as paclitaxel or docetaxel and platinum drugs such as carboplatin and cisplatin (Torrisi *et al*, 2008).

Studies have suggested that patients with TNBC may benefit from high-dose or intensified chemotherapy (weekly or bi-weekly) regimens (Gluz *et al*, 2008). Dose-intensive chemotherapy may result in higher side effects compared to the less-intensive regimens. Side effects generally include alopaecia, neutropaenia and diarrhoea. A *p53*-based cyclotherapy approach could be applied in this setting to continue intensified treatments to tackle aggressive *p53*-mutant TNBC. While using *p53* activators for the purposes of cyclotherapy, a major consideration has to be p53-related toxicities to normal tissues. Past studies using preclinical models have indicated specific toxicity only in tissues harbouring wild-type p53 during cytotoxic therapy (Komarov *et al*, 1999). In order to enhance the therapeutic window using cyclotherapy, specific and highly selective *p53* activators that can induce a mild 'cytostatic' effect will need to be carefully selected.

FUTURE DIRECTIONS

A remarkable *in vivo* study showing a reduction in neutropaenia in mice after pretreatment with nutlin-3 provides great optimism for p53-based cyclotherapy (Sur *et al*, 2009). However, Nutlin-3 has yet to be clinically approved. A nutlin-like compound, RG7112, has been tested in patients with mdm2-amplified liposarcomas (Ray-Coquard *et al*, 2012). While one patient achieved partial remission and 14 stable disease among the 20 treated, this study also highlighted that all of the patients who received neoadjuvant therapy with RG7112 experienced severe side effects including neutropaenia. These side effects may have been observed perhaps due to the high doses ($1440 \, \mathrm{mg \, m^{-2} \, day^{-1}}$) used. Based on these observations, it cannot be ruled out that there may be many limitations for nutlin use in the clinic for the purposes of cyclotherapy.

Recently, Brown *et al* (2013) have developed stapled peptides that are highly specific inhibitors of Mdm2/MdmX. These compounds are more potent in activating *p53* than peptides derived from a wild-type *p53* sequence. Unlike nultin-3, these peptides can sustain p53 activity over a wider range of concentrations without much toxicity to the cells. This suggests that these peptides could be ideal candidates for future cyclotherapy.

ActD, unlike nutlin-3, is a clinically approved drug with promise for cyclotherapy along-side S-phase but not mitotic poisons (Rao *et al*, 2010; van Leeuwen *et al*, 2012). A recent investigation of 16 different cyclotherapy regimes using nutlin-3, ActD, leptomycin B and tenovin 6 as cyclotherapeutic drugs in combination with the S-phase poisons, gemcitabine and cytosine arabinoside and the M-phase poisons, vinorelbine and vinblastine, highlights the need

to assess both short-term and long-term effects of these combinations (van Leeuwen *et al*, 2012).

Approval of cyclotherapy in the clinic requires the development of *in vivo* models. We are investigating the effects of ActD *in vivo* to deduce the interval of time during which growth arrest can be established in mice gut crypt cells. This interval may include a window of opportunity to add an S-phase-specific drug that can then specifically target only tumour cells sparing the cells of gut crypts, one target of conventional chemotherapy approaches. It is critical to test the cyclotherapy combinations on different normal tissues to understand tissue-specific pathological effects. The p53 activators currently being tested for cyclotherapy all have distinct mechanisms to activate p53 and need to be tested in different combinations *in vivo*. The future of cyclotherapy holds exciting promise for targeting cancer cells while sparing normal tissues in a way currently not achieved with conventional chemotherapy.

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