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Review

Improving cancer therapy by non-genotoxic activation of p53

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Abstract

Inactivation of p53 function is a common event in cancer. Approximately 50% of human tumours express mutant p53 and there is evidence that in others, including many childhood tumours, p53 function is impaired in other ways. These defects in p53 function may be due to the alteration of cellular factors that modulate p53 or to the expression of viral oncoproteins. Radiotherapy and many of the chemotherapeutic drugs currently used in cancer treatment are potent activators of p53. However, most of these therapies have a serious drawback, and that is the long-term consequences of their DNA damaging effects. Here, we review the discoveries in p53 research that are most significant to the development of new therapies based on the induction of the transcriptional activity of p53 in a non-genotoxic way and discuss the situations in which this type of approach may be most beneficial.

Keywords: Non-genotoxic; p53; Mdm2; E6

1. Major discoveries in the p53 field

A lot of effort has been expended to try to reactivate mutant p53, to introduce p53 in cells lacking functional protein or to exploit the lack of active p53 in tumour cells. This is a great challenge which has led to a variety of very exciting approaches which are discussed elsewhere in Refs. [1–3]. Here, we have focused on those cancers that express wild-type p53, but where its function is impaired due to defects in its regulation. Below we have summarised what we consider to be the discoveries in p53 research that are most significant to the development of new ways to potentiate its tumour suppressor function in these kind of tumours.

1.1. p53 tumour suppressor activity is impaired in most cancers

In 1979, it was discovered that T antigen binds to a host protein with an apparent molecular weight of 53/54 kDa in SV40-transformed cells [4,5]. For a long time, the *P53* gene was thought to be an oncogene and it was not until 10 years later that p53 was found to be mutated in diverse human tumours [6,7], suggesting its role as a tumour suppressor. At the same time, two laboratories reported that wild-type p53 can act as a suppressor of transformation by mutant p53 and oncogenes [8,9]. In 1992, Donehower and colleagues described that *P53* knock-out mice have a higher propensity to develop tumours spontaneously [10]. This work firmly established p53's role as a tumour suppressor. Supporting this evidence on p53's tumour suppressor role in humans, the Li–Fraumeni cancer predisposition syndrome was found to be associated with germline *P53* mutations [11].

Approximately 50% of human tumours carry inactivating mutations in the *P53* gene and thorough analysis of the complete *P53* sequence and activity is revealing that this percentage could be significantly higher [12]. Furthermore, in many of the tumours that encode wild-type p53, its levels and activity are hampered due to alterations in other cellular factors or the expression of viral oncogenes (see below).

Tumours encoding wild-type p53 are thought to respond better to radiation and current chemotherapies than tumours lacking wild-type p53 and lose their responsiveness when the p53 is mutated [13]. However, as recently described, this is not a general rule, since in the case of advanced breast cancer tumours treated with epirubicin and cyclophosphamide [14] and ovarian can-

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cers treated with cisplatin and paclitaxel [15], a positive association between *P53* mutation and the success of the treatment has been reported. This dispute on the influence of p53 status with regard to patient outcome may reflect that the response is probably dependent on the type of tumour as well as on the dosage of the drugs.

1.2. p53 functions primarily as a transcription factor that triggers cell-cycle arrest or apoptosis

The tumour suppressor activity of wild-type p53 is mainly due to its ability to act as a transcription factor and induce the expression of an ever growing number of proteins [16–18]. Some of these proteins are involved in causing the inhibition of cell proliferation by promoting cell cycle arrest at different checkpoints and others can cause the induction of cell death by apoptosis. Accordingly, mice bearing a transcriptionally inactive mutant of p53 are prone to tumours [19], indicating that the transcription activity domain of p53 is necessary for its tumour suppressor function.

The discovery of p53 alterations that modulate its effects on different promoters and the definition of cofactors that specifically promote p53-induced apoptosis suggest that the apoptotic and cell-cycle arrest activities of p53 can be separated. In an insightful and recent review, these findings are thoroughly discussed [17].

1.3. Regulation of p53 levels: Mdm2 plays a crucial role

If p53's primary function is to stop cell proliferation leading to a cell-cycle halt or to the induction of cell death by apoptosis, its levels and activity must be tightly regulated.

The mechanisms that control *P53* gene transcription remain largely unknown and many reports have indicated that p53 levels are mainly regulated at the post-transcriptional level. However, recent work suggests the involvement of *P53* promoter activation by oestrogen [20] as well as by p53 itself and Nuclear Factor Kappa B (NFkB) [21] and the existence of a promoter element that is involved in basal *P53* gene expression and the stress response [22].

In normal, non-stressed cells, p53 has a very short half life due to the following autoregulatory feedback loop mechanism in which the Mdm2 protein plays a key role (Fig. 1). Wild-type p53 acts as a transcriptional activator of the *Mdm2* gene [23]. In turn, Mdm2, which itself has a very short half-life due its autoubiquitination activity, has the ability to interact with p53 and to function as a ubiquitin E3 ligase that promotes the conjugation of p53 to polyubiquitin [24]. This conjugation to ubiquitin serves as a tag that effectively targets p53 for degradation by the proteasome [25,26]. In this way, in normal, non-stressed cells, p53 levels are maintained at low level and cells are allowed to proliferate. Contributing to its 'anti-p53' function, Mdm2 is also thought to impair the transcriptional activity of p53 by masking the transactivation domain of this tumour suppressor. Another degree of complexity has arisen from the observation that Mdm2 can increase the translation of p53 as well as of a p53 truncated form with altered transcriptional activity [27].

Supporting Mdm2's crucial role in the regulation of p53 activity, *Mdm2* knockout mice are rescued from embryonic lethality by deletion of *P53* [28]. A similar requirement has also been observed when *Mdmx* (*Mdm4*) knockout mice were developed, suggesting the importance of the MdmX protein in regulating the levels and activity of p53 [29,30].

In tumour cells that encode mutant p53, these forms are still susceptible to degradation by Mdm2; however, the ability to act as a transcriptional activator is generally abolished and, therefore, the levels of Mdm2 are decreased giving rise to the accumulation of p53 in the cells [31]. This is why the detection of high levels of p53 in tumours can be used as an indicator (but not as full proof) of the existence of mutations in the *P53* gene.

1.4. p53 function is hindered in a wide variety of tumours in which P53 *is not mutated*

In many cancers where the *P53* gene is intact, its tumour suppressor function is thought to be blocked by the overexpression or inactivation of cellular factors that regulate the levels and activity of p53 or by the expression of certain oncoviral proteins (Fig. 2). These include the overexpression of the Mdm2 protein [32], defects in the expression of the p14ARF tumour suppressor [33], mutations in kinases such as ATM or Chk2 [34,35], chromosome translocations involving the PML or the nucleophosmin proteins [36,37] or the infection with certain viruses.

Papovaviruses (papillomaviruses and polyomaviruses), hepadnaviruses, retroviral oncoviruses and adenoviruses encode proteins that cripple the retinoblastoma (Rb) and the p53 pathways [38,39]. In humans, one of the most important and best studied cases is that of the malignant strains of the human papillomavirus (HPV). As shown in 1990, the HPV protein E6 forms a complex with p53 and promotes its degradation through the ubiquitin pathway which involves the E6AP ubiquitin E3 ligase (reviewed in Ref. [40]). Similarly, the adenoviral proteins E1B55kD and E4orf6 effectively target p53 for degradation (reviewed in Ref. [41]). Other human tumour viral proteins that bind to p53 include the hepatitis B virus X antigen [42], Kaposi's sarcoma-associated herpesvirus protein LANA-1 [43], the Epstein–Barr virus EBNA-5 protein [44] and the human hepatitis C protein NS5A [45]. The human T-lymphotropic virus type I (HTLV-1) Tax protein interferes with the transcriptional activation of

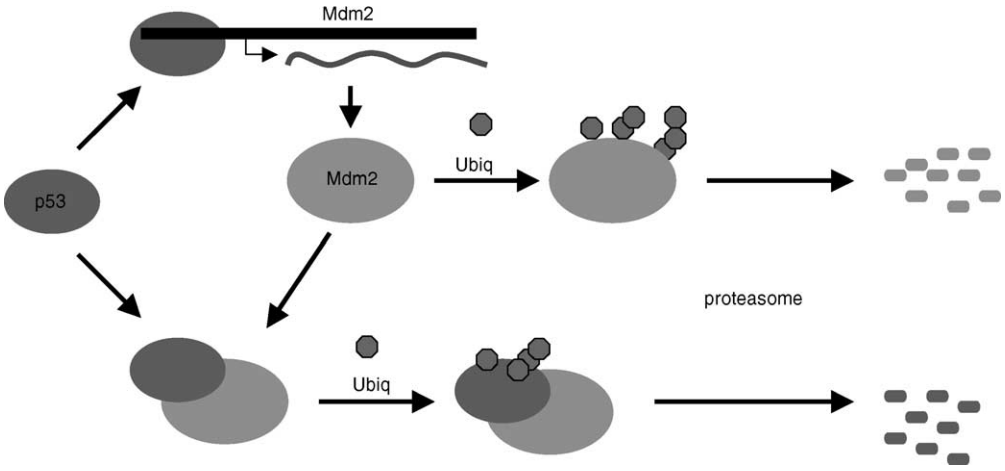

Fig. 1. p53/Mdm2 feedback loop mechanism.

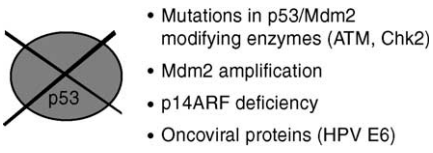

Fig. 2. Events leading to an impaired p53 function in tumours expressing wild-type p53.

p53 through mechanisms that may involve binding to CREB binding protein (CBP), hyperphosphorylation of p53 at Ser-15 and activation of NF-κB [46].

1.5. p53 can be activated by DNA damage

It is well known that p53′s levels and transcriptional activity increases in cells that are irradiated or treated with DNA damaging agents. To verify this discovery *in vivo*, Prof. Peter Hall irradiated the skin of his arm with ultraviolet (UV) light. Biopsies of the skin in the UV-treated regions unequivocally showed an increase in the p53 levels in the nucleus of the cells [47]. Supporting that this activation is, at least in part, due to the appearance of DNA lesions, microinjection of cells with a restriction enzyme could induce the accumulation of p53 [48]. Telomere erosion during the ageing process and defects in the DNA structure due to mistakes during the processes of DNA replication and recombination are thought to trigger the p53 response by mechanisms that are likely to be similar to the response to DNA damaging agents [49].

The elucidation of the mechanisms by which p53 is activated when cells are subjected to stress has been an area of intense research. In response to DNA damage, the activity of certain kinases (such as DNA-dependent protein kinase (DNAPK), ATM) may be increased and p53 and/or Mdm2 are modified by phosphorylation. In some cases, these phosphorylation events have been suggested to inhibit the ability of p53 and Mdm2 to interact with each other or to inhibit the ability of Mdm2 to carry out its function. Important findings on the induction of p53 and Mdm2 posttranslational modifications have been summarised and discussed extensively [50–55]. Other modifications of p53 and Mdm2 that may affect p53's stability and activity include small ubiquitin-related polypeptide (SUMO) conjugation and acetylation [56,57].

1.6. p53 can be activated without causing DNA damage

p53 activity can also be increased by a variety of stresses that do not, at least directly, involve DNA damage. These include hypoxia, serum starvation, heat, cold, pH, ribonucleotide depletion, glycerol and the inhibition of nuclear export [58–61]. Pioneering clinical trials support the value of these observations [58,62].

Oncogenic signals and certain viral oncoproteins can also increase the levels of p53 [63]. This activation, at least in some situations, is mediated by an increase in the levels of expression of the ARF tumour suppressor, an antagonist of Mdm2 function. An increase in ARF levels causes the inactivation of the Mdm2 E3 ubiquitin-ligase function and therefore a decrease in the levels of ubiquitinated p53 [64,65] which is also accompanied by an enhancement of its transcriptional activity.

2. Do normal cells and tumour cells respond differently to the activation of p53?

The cell cycle is a tightly controlled process with many checks and balances to make sure that only healthy cells divide and that they divide correctly. Cancer cells have escaped the rigorous controls of this process resulting in unlimited cell and tissue growth in the wrong places. In tissue culture conditions, many tumour cells are very susceptible to apoptosis in response to the activation of p53. This is probably due to alterations in the cell-cycle checkpoints in these cells and possibly to their high

levels of E2F, which are necessary for the entry of cells into the S-phase of the cell cycle. It is well documented that simultaneous expression of E2F and p53 increases cell death by apoptosis [66]. This impaired functionality may explain why, in tumour cells, p53 activation frequently results in high levels of cell death by apoptosis.

Instead, certain types of normal cells, like fibroblasts, which tend to respond to stress by stopping their proliferation, are relatively resistant to the effects of p53 activators [61,67,68]. Furthermore, p53 can play a protective role against UV-induced apoptosis and chemotherapeutic drugs [69–72]. Therefore, chronic stress activation of p53 could protect cells from a subsequent stronger insult, a concept termed as hormesis [73]. But growth arrest is not the most likely response to p53 activation in many other normal cell types, which like T-lymphocytes are highly susceptible to apoptosis. Therefore, dissecting the mechanisms that exclusively trigger an apoptotic response from those that elicit growth arrest may help us predict the response of a particular tumour to therapy [17,74].

The induction of the p53 response in some normal tissues is thought to be at least partially responsible for many of the adverse side-effects of current chemotherapeutic drugs, such as gastrointestinal dysfunction or hair loss. Accordingly, Donehower and colleagues [75] have observed that a constitutive increase in p53 activity is associated with the appearance of very clear symptoms of premature ageing in mice. This demonstrates that an uncontrolled increase in p53 activity has serious drawbacks. A set of results that are more encouraging come from the observation made in M. Serrano's laboratory [76]. This work shows that mice with an extra copy of the *P53* gene are protected from tumorigenesis, but unlike the mice developed in the Donehower laboratory, do not show early ageing symptoms. The difference between the phenotype observed by Donehower and colleagues and the phenotype of the mice with three copies of TP53 is very likely to be due to the possibility that in the latter case, p53 levels are properly regulated. These results indicate that a general increase in p53 levels causes premature ageing and that only if p53 is susceptible to efficient regulation, can this be avoided. But could the picture become brighter if the effect of p53 was reversible? It is clear that people can recover to some extent from some of the premature ageing-like secondary effects of chemotherapy such as hair loss. Additionally, there is some experimental evidence showing that cultured cells can recover after a transient induction of the p53 response [60,61,77,78]. If the effects of transient p53 induction in normal tissues are reversible, there is obviously an opportunity, and 'all we have to do' is find potent non-genotoxic drugs with lower risk long-term effects.

In summary, as with all therapies, when dealing with the exploitation of the p53 pathway to develop anti-cancer treatments, the issue of specificity arises. That is, is it possible to effectively kill tumour cells by inducing the p53 response without inducing intolerable levels of cell death or ageing in normal tissues? Are these ageing effects irreversible? Additionally, if p53 elicits a cytostatic response in a given tumour, will it protect some malignant cells from the killing effect of therapeutic agents? These questions are very difficult to answer in a general way and will depend on the characteristics of each type of cancer. Clearly, the development of effective p53-dependent therapeutic agents may be tightly linked to the improvement of specific drug delivery systems. However, the discovery of non-DNA damaging p53 activators may provide substitutes for the drugs currently used or at least provide agents that can effectively sensitise tumour cells to the effects of currently used therapies.

Alternatively, one could think of taking a radically opposite approach and aim to decrease the devastating effects of p53 induction in normal tissues and therefore improve quality of life of patients during treatment. Drugs like the p53 inhibitor pifithrin described by A. Gudkov's laboratory provide an example of this line of reasoning [79].

3. How can we improve present treatments?

Contrary to the findings in solid tumours occurring in adults, where approximately 50% of the tumours bear *P53* mutations, the rate of *P53* mutations in haematological malignant diseases and childhood cancers is significantly lower [80,81]. This may be the key to the much better prognosis of children with cancer. However, the DNA damaging effects of current therapies is especially important to bear in mind when considering the treatment of young patients because it is emerging that the damage induced by these treatments may lead to growth and development problems in children, infertility, and second malignant tumour induction later on in life [82–87]. These secondary tumours may not necessarily derive from the original cancerous cells, but from normal cells that were damaged during treatment. This is why the search for novel non-genotoxic activators of the p53 response is thought to be essential in improving the treatment of those cancers in which p53 function is not abolished by mutation. So, even if we do not solve the problem of specificity, we can at least try to decrease the long-term side-effects of current treatments or at least the risk of second tumours.

3.1. Inhibiting the effect of Mdm2 on p53

How can we activate p53 in a non-genotoxic way? If Mdm2 is an important inhibitor of p53 function in cells, a likely way to increase p53 levels is by impairing its function. In recent years, considerable efforts have been

made to dissect the p53/Mdm2 feedback loop mechanism. This work has given rise to several ideas on how to impair Mdm2 function. We can decrease the effects of Mdm2 on p53, by decreasing its expression with antisense RNAs [88] and transcription inhibitors [89] or by inhibiting the interaction between p53 and Mdm2 [90].

Inhibiting the activity of the proteasome very effectively increases p53 levels and encouraging results are being obtained in clinical trials with the proteasome inhibitor PS-341 [91]. However, whether proteasome inhibitors are effective at inducing p53-dependent transcription has not been fully established and preliminary results indicated that these agents do not cause cell death in a p53-dependent way [93].

Inhibiting ubiquitination of p53 by Mdm2 may also be a suitable approach. In this sense, the suggestion that accumulation of ubiquitinated forms of p53 and of Mdm2 autoubiquitination are separable is of significant importance [65]. If p53 and Mdm2 are ubiquitinated by distinct mechanisms, it may be possible to specifically inhibit the degradation of p53 complexes without the drawback of accumulating Mdm2 simultaneously. Several compounds have been shown to specifically inhibit p53 ubiquitination by Mdm2 without affecting Mdm2's ubiquitination [92].

A very effective alternative is to induce the accumulation of p53 in the nucleus, where it can act as a transcriptional activator more effectively. The nuclear export inhibitor leptomycin B (LMB) has been shown to be an extremely potent activator of p53 transcriptional activity [93,94]. Additionally, unlike other potent inducers of the p53 response such as actinomycin D and the protein kinase inhibitor H7, LMB has a relatively mild and reversible cell-cycle arresting effect on normal human fibroblasts at micromolar concentrations, but effectively induces cell death in cells derived from neuroblastomas, one of the most frequent solid tumours occurring in children [67]. However, despite the promising properties of LMB in cell culture systems, the pharmacokinetic behaviour of this drug is difficult to study. This together with the secondary effects observed when the drug was injected intravenously has stopped tests in humans [95]. Nevertheless, we reasoned that this drug could be of interest in the case of tumours that can be treated or prevented by topical application.

3.2. Inhibiting the effect of E6 on p53 in HPV-infected cells

One type of tumour for which topical application may be greatly beneficial is HPV-positive cervical cancer, in which the activity of p53 is rarely abolished by mutation, but is instead effectively decreased by the action of the oncoviral protein E6 [96]. HPV infects the basal cells of the epithelia including genital and anal areas, and HPV DNA has been found in 90% of cervical cancers and 50% of vulvar cancers [97]. Since HPV infection is initially asymptomatic, despite advances in screening, cervical cancer is the second most common cause of cancer-related death in women worldwide, in some developing countries accounting for the highest cancer mortality [98].

The study of the function of HPV proteins has been crucial in understanding the oncogenic process it induces. The sequence of events by which HPV disturbs the normal terminal differentiation process of cervical cells, is essentially as follows. As the viral DNA integrates into the genome, the expression of two viral products, E6 and E7 is enhanced. E7 binds the retinoblastoma gene product whereas E6 mediates the degradation of p53. As a result, crucial cell cycle check points are compromised and the cell can be immortalised.

It is important to underscore that the current forms of treatment may at best prevent the progression of dysplasia to cancer, but none of these treatments are a cure of the virus infection. Therefore, prevention and regular screenings are the only way to 'cure' HPV infection [97]. Several forms of HPV vaccines, some of which may have therapeutic as well as preventative activities are being developed: however, years of clinical trials are still required before they can routinely be used. Indeed, ethical issues may prevent widespread regular screening and vaccination being adopted in many countries.

Although treatment of the dysplasia varies between institutions, patients with cervical intraepithelial neoplasia may be treated by cryosurgery, laser ablation or removing a wedge of the cervix by surgical excision. For patients with a locally advanced stage of disease, failure to obtain regional control usually results in death. It is in these cases that the search of new agents that selectively kill HPV-infected cells, and ideally in a non-genotoxic way, is of great interest. Medical treatments that include the use of topical 5-fluorouracil, interferon and Imiquimod have shown some activity. Systemic chemotherapy is used primarily to treat advanced or recurrent cervical cancers [99]. Some of the drugs with activity against cervical cancers are: cisplatin, ifosfamide, dibromodulcitol, doxorubicin, hydroxyurea, 5-fluorouracil, mitomycin, vincristine and bleomycin. Although the use of concurrent chemotherapy and radiation therapy has resulted in a significant increase in patient survival, the results obtained can be qualified as moderate and treatments are highly genotoxic.

As described above, it has been demonstrated that the nuclear export inhibitor LMB is an extremely potent activator of the p53 response in a variety of cells in culture. These results led us to test whether this drug was able to stabilise p53 and induce its transcriptional activity and growth inhibitory functions in HPV-positive cells derived from cervical cancers. As shown in our laboratory [100], LMB was able to increase p53 levels and activity and to significantly potentiate the apoptotic effects of low con-

centrations of actinomycin D in these cells. Furthermore, by introducing the dominant-negative form of p53 in these cells, the killing effect of the drug combination (LMB + Actinomycin D) at nanomolar concentrations was shown to be substantially p53-dependent. This study has two consequences. Firstly, it shows that HPV cells are highly sensitive to the activation of p53 and secondly, it suggests that it is possible to activate p53 in these cells in a non-genotoxic way. In summary, we consider that due to their growing medical importance and the possibility to treat them topically, HPV-related malignancies are ideal to test novel non-DNA damaging activators of the p53 tumour suppressor function.

4. Concluding remarks

The vast number of reports dealing with the p53 tumour suppressor is indicative of the resources invested in p53-related research. But how is this effort helping in cancer treatment? To try to answer this question, we have reviewed the major achievements in the field and attempted to evaluate the pros and cons of exploiting the p53 pathway to search for novel cancer treatments. In particular, we have tried to assess how far we can use our knowledge on the regulation of the p53 tumour suppressor function to develop new cancer therapies to treat those tumours in which p53 is not mutated, which include most childhood cancers and cancers associated with viral infections. In summary, we conclude that p53 research is contributing new ideas to the development of novel agents, which at least may have less harmful long-term secondary effects than the ones currently in use. The purpose of this review was to stimulate researchers to keep on discovering new suitable targets of the p53 pathway and to encourage the search for novel non-genotoxic activators of p53 by high-throughput screenings.

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