PERGAMON

European Journal of Cancer 39 (2003) 1828–1834

European Journal of Cancer

www.ejconline.com

Current Perspective

Small molecules that reactivate mutant p53

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Received 22 April 2003; accepted 25 April 2003

Abstract

Around half of all human tumours carry mutant p53. This allows escape from p53-induced cell cycle arrest and apoptosis. Many tumours express mutant p53 proteins at elevated levels. Restoration of wild-type p53 function should trigger massive apoptosis in tumour cells and thus eradicate tumours. Various types of small molecules have been identified that can restore native conformation and wild-type function to mutant p53. Such molecules may serve as leads for the development of novel efficient anticancer drugs.

Keywords: Mutant p53; Cancer; Small molecules; p53 Reactivation; Apoptosis; Novel cancer therapy

1. Introduction

The *p*53 tumour suppressor gene is inactivated by point mutation in around 50% of all human tumours (see http://www.iarc.fr/p53). Such a high mutation frequency indicates a strong selection for loss of normal p53 function during tumorigenesis. Moreover, it is becoming increasingly clear that *p*53 mutations may not only disrupt normal p53 function, but also endow mutant p53 with dominant-negative and/or dominant gain-of-function activity. The wild-type p53 protein binds specifically to DNA and regulates the expression of a set of target genes, perhaps as many as several hundred or more. Transactivation of genes, such as *p*21 and Growth Arrest and DNA Damage 45 (*GADD45*), blocks cell cycle progression while transactivation of *Bax*, *Fas*, *Noxa*, p53 Upregulated Modulator of Apoptosis (*PUMA*) and other genes promotes cell death by apoptosis. In addition, p53 can repress transcription of certain genes, including *Bcl*-2, the Insulin-like Growth Factor I (*IGF1*) receptor, Microtubule Associated protein 4 (*MAP4*), *and the catalytic subunit of human telomerase (hTERT)* (Fig. 1; see Ref. 1 for a review). One of p53's target genes, *MDM2*, encodes a protein that binds p53 and catalyses proteasome-mediated p53 degradation, forming a negative feedback loop that controls p53 levels. Under normal conditions, wild-type p53 is expressed at low levels in most tissues. Mutant p53 proteins fail to bind DNA and transactivate p53 target genes, and thus fail to induce the p53-antagonising MDM2 protein. This leads to elevated levels of mutant p53 in many tumours.

The frequent mutation of *p*53 in human tumours and the presumed dominant gain-of-function effect of *p*53 mutations makes mutant p53 a prime target for pharmacological therapeutic intervention in cancer. The fact that this target is often expressed at high levels in tumours, but not in normal cells and tissues, should provide a necessary selectivity for tumour cells. Such selectivity is also expected to arise from p53-activating post-translational modifications in tumour cells, e.g. phosphorylation, and synergising proapoptotic genetic and epigenetic alterations in tumour cells, for instance dysregulation of the pRb pathway. Thus, pharmacological reactivation of mutant p53 should efficiently eliminate tumour cells through induction of apoptosis with minor unwanted side-effects.

Over the past few years, a number of small molecules that reactivate mutant p53 have been identified and characterised. In this review, we will describe some of these molecules and discuss possible mechanisms for mutant p53 rescue.

2. Short synthetic peptides

Previous studies in several laboratories demonstrated that short synthetic peptides derived from the

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0959-8049/03/$ - see front matter © 2003 Published by Elsevier Ltd.
doi:10.1016/S0959-8049(03)00454-4

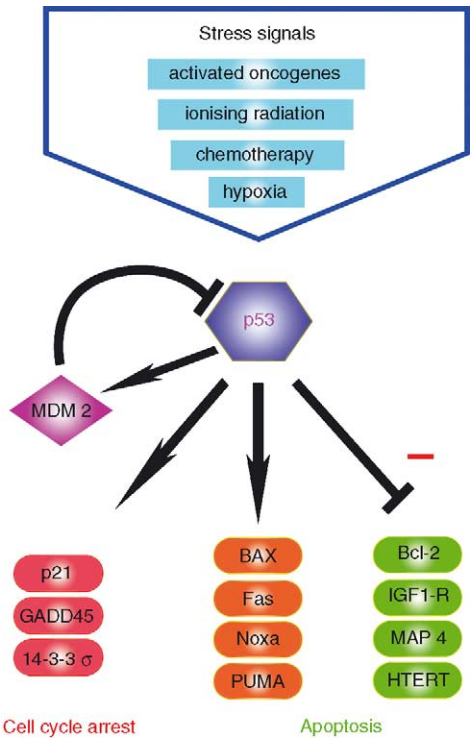

Fig. 1. The p53 pathway. Stress signals such as activated oncogenes, ionising radiation, chemotherapeutic drugs, and hypoxia, trigger p53 protein accumulation and p53-dependent transactivation of target genes that induce cell cycle arrest or apoptosis, and transrepression of certain anti-apoptotic genes. p53 also transactivates the *MDM2* gene whose protein product binds p53 and targets p53 for proteasome-mediated degradation, forming a negative feedback loop that controls p53 levels.

p53 C-terminal region and monoclonal antibodies that recognise a C-terminal epitope in p53 can stimulate specific DNA binding of wild-type p53 *in trans*. Interestingly, such peptides were also shown to increase the DNA binding and transcriptional transactivation activity of mutant p53 proteins *in vitro* and in living cells. Moreover, peptide 46, corresponding to residues 361–382 in human p53, induced apoptosis when introduced in human tumour cells carrying mutant p53 [2]. This peptide was shown to bind to the p53 core domain, indicating that it may directly restore proper core domain conformation and/or DNA binding [3].

More recent studies have identified a short peptide that interacts with the p53 core domain and stabilises the folding of mutant p53. The p53-interacting ASPP protein (previously 53BP2) binds to the p53 core domain and enhances p53-mediated transactivation, specifically stimulating the apoptotic function of p53 [4]. A nine-residue peptide, CDB3, was developed based on the crystal structure of the complex between the p53 core and ASPP/53BP2 [5]. CDB3 binds to the core domain and stabilises the structure of p53 mutant proteins as measured by nuclear magnetic resonance (NMR) [6]. The rationale is that a peptide which binds with higher affinity to a properly folded state than to an unfolded form of the mutant will shift the equilibrium towards the active wild-type conformation. Despite its binding to the edge of the DNA-binding domain, CDB3 does not interfere with p53's DNA binding. CDB3 induced the refolding of the denatured p53 core domain, as well as restoration of the sequence-specific DNA binding to various p53 mutants, including the highly destabilised Thr-195 mutant. Based on these results, CDB3 was suggested to act through a chaperone mechanism, stabilising mutant p53 in a bioactive conformation. The CDB3 peptide could be exchanged for the tighter-binding cognate DNA, thus leading to an active p53-DNA complex. CDB3 was also shown to restore wild-type conformation and transcriptional activity to two hot-spot p53 mutants, His-175 and His-273, in human tumour cells (Selivanova and colleagues, unpublished results).

3. Chemical chaperones

Certain low molecular weight compounds, including glycerol, trimethylamine *N*-oxide, and deuterated water, can stabilise proteins against thermally induced denaturation *in vitro* [7]. Glycerol might increase the relative hydration around a protein, resulting in a decreased relative surface area of the polypeptide. This could lead to tighter packing of the protein and in turn enhanced stability. Incubation of cells carrying mutant p53 with glycerol was shown to restore the wild-type conformation, DNA binding and transcriptional transactivation function to mutant p53, and induce growth arrest or apoptosis, depending on the cell type. Unfortunately, the concentrations of glycerol required for mutant p53 rescue are very high, 0.6 M, and so this approach is unlikely to be translated to clinical cancer therapy [8,9]. However, it may still be useful as a model system to study the requirements for the correction of folding defects of mutant p53.

4. Ellipticine

The National Cancer Institute has established a database with information about the activity of more than 70 000 low molecular weight compounds against a panel of 60 human tumour cell lines of different origin (http://dtp.nci.nih.gov). Information about the status of *p53* and other cancer-related genes in these lines is available. Analysis of this database has revealed that a group of molecules called ellipticiniums show preferential activity towards mutant *p53*-carrying tumour cell lines of the colon, central nervous system (CNS), and kidney origin [10]. Subsequent studies by others have shown that the related compound 9-hydroxy-ellipticine (9HE) causes

cell cycle arrest and apoptosis in human tumour cells carrying various forms of mutant p53 [11]. Furthermore, 9HE induced mRNA levels of two p53 target genes, *p21* and *Bax*, in mutant p53-expressing tumour cells, but not in the corresponding p53-null cells. These studies suggest that certain ellipticines may restore wild-type function to mutant p53. However, the mechanism of action of 9HE remains to be determined, as well as its ability to suppress tumour growth *in vivo*.

5. CP-31398

In order to identify small molecules that can rescue mutant p53, Foster and colleagues [12] screened a library of 100 000 chemicals for compounds that could stabilise the native conformation of the wild-type p53 core domain upon thermal denaturation. Samples were heated in the presence of compounds and p53 conformation was assessed by the conformation-specific antibodies PAb1620 and PAb240 that recognise the folded and unfolded conformations of p53, respectively. The most promising molecule, CP-31398, enhanced the stability of Ala-173 and His-273 mutant p53 core domains *in vitro*, restored native conformation and transcriptional transactivation activity to Ala-173 mutant p53 in living cells, and caused the induction of the p53 target gene *p21*. Moreover, CP-31398 inhibited the growth of human melanoma and colon carcinoma cells carrying p53 mutated at position 249 or 241, respectively, in nude mice. No toxicity was observed at the therapeutic doses *in vivo* [12].

The exact mechanism of CP-31398 remains unclear. The compound could interact directly with already synthesised p53 or mediate p53 folding during biosynthesis. The first attempt to detect physical interactions between p53 and CP-31398 was unsuccessful. NMR showed that CP-31398 does not bind to the core domain of recombinant wild-type or mutant p53. Rather, gel shift assays and NMR indicated that the compound binds DNA. Some experimental evidence suggests that CP-31398 can affect the folding of newly synthesised p53 only, which could explain the negative NMR results [13].

Further studies have confirmed that CP-31398 treatment causes the induction of p53 target genes like *p21*, *MDM2*, *Bax* and *KILLER/DR5*, and apoptosis-related changes at the protein level such as cleavage of Poly ADDRibose Polymerase (PARP). The effect of CP-31398 seems to be cell line-dependent, since the p53 targets *Bax* and *MDM-2* were induced in SKOV cells carrying Tet-regulated exogenous His-175 mutant p53, but not in other lines expressing endogenous mutant p53, including DLD1, SW480 and SKBr3 [13,14]. CP-31398 did not induce mutant p53-dependent apoptosis, but rather appeared to affect cells irrespective of their p53 status [14]. CP-31398 induced apoptosis in HCT116 cells carrying wild-type *p53*, but not in the isogenic *p53*-null line in which wild-type *p53* has been knocked out [15]. The compound caused increased wild-type *p53* levels and activated some p53 target genes such as *p21* and *Bax* in HCT116 cells, but p21 upregulation was also observed in *p53*-null HCT116 cells, although to a lesser extent. Induction of Bax resulted in cytochrome C release in a wild-type p53-dependent manner. CP-31398 induced activation of caspase-9 and caspase-3, resulting in PARP cleavage in a wild-type p53-dependent manner, but no changes in Apaf-1 protein levels were detected [15]. CP-31398 potentiated ultraviolet (UV)-induced apoptosis via the Bax/mitochondria/caspase-9 pathway in melanoma cells expressing wild-type p53 [16].

CP-31398-mediated upregulation of wild-type p53 levels and transactivation of downstream p53 targets could be due to the induction of DNA damage, as NMR studies have indicated that the compound is a DNA intercalator. Yet p53 phosphorylation at Ser-15 and Ser-20, often associated with DNA damage, has not been observed after treatment with CP-31398 [17]. Instead, CP-31398 was shown to inhibit the ubiquitination of wild-type p53, which may explain its ability to induce p53 accumulation. The interaction between p53 and MDM-2 was not affected by CP-31398 [17].

Mutant p53 rescue by CP-31398 may act synergistically with currently used chemotherapeutic drugs, which often show a better efficacy against wild-type p53-carrying tumours. The combination of CP-31398 with cisplatin, doxorubicin (adriamycin), or VP-16 resulted in an additive effect in cell cultures [14], suggesting that CP-31398 could be an important adjuvant treatment, particularly for tumours carrying wild-type p53.

Thus, CP-31398 has promising activity on mutant and wild-type p53 protein *in vitro* and in cellular assays, but p53-independent cellular toxicity may limit its use in the clinic. Further studies should aim at identifying CP-31398 analogues with a lower toxicity and higher selectivity for mutant p53-expressing tumour cells.

6. WR1065

The aminothiol WR1065 is the active metabolite of amifostine, an agent used clinically to reduce the side-effects of chemotherapy and radiotherapy. Amifostine protects cells from DNA damage induced by irradiation, with strong specificity for normal cells, but does not affect the killing of cancer cells. WR1065 was shown to restore wild-type conformation to the temperature-sensitive Met-272 mutant p53 in the oesophageal carcinoma cell line TE-1 and stimulate its DNA binding at a concentration of 1–4 mM. This was followed by transactivation of the p53 target genes *p21*, *GADD45* and *MDM2*, and G1 cell cycle arrest [18]. Subsequent stud-

ies demonstrated that WR1065 induces wild-type p53 accumulation in MCF-7 cells through a JNK-dependent and DNA damage-independent pathway that leads to p53 phosphorylation at Thr-81 and escape from proteasome-mediated degradation [19]. WR1065 was further shown to reduce p53 and enhance its redox-dependent DNA binding [20]. It remains to be determined whether WR1065 can rescue other mutant forms of p53.

7. PRIMA-1

The low molecular weight compound PRIMA-1 was identified in a cellular screen of a chemical library from the National Cancer Institute (http://dtp.nci.nih.gov), using Saos-2-His-273 osteosarcoma cell line that expresses Tet-regulated mutant p53 [21]. Compounds that suppressed the growth of cells expressing mutant p53, but did not affect the same cells in the absence of mutant p53 expression (in the presence of doxycycline) were selected for further study. Mutant p53-dependent induction of apoptosis by PRIMA-1 was confirmed in a panel of cell lines expressing different Tet-regulated p53 mutants. Gel shift assays demonstrated that PRIMA-1 stimulated DNA binding of a wide range of mutant p53 proteins. The only exception was the Phe-176 mutant p53 in which a Cys residue at position 176 that holds a Zn atom responsible for shaping the DNA binding domain is lacking [22]. The Phe-176 mutation results in a severely distorted protein structure that is probably refractory to refolding.

It is noteworthy that restoration of wild-type p53 conformation and DNA binding by PRIMA-1 was more efficient in cellular extracts compared with recombinant proteins. This suggests that specific post-translational modifications such as phosphorylation and acetylation, which are not introduced during biosynthesis in bacteria, are important for PRIMA-1-mediated reactivation of mutant p53. Alternatively, mutant p53 rescue by PRIMA-1 may require an intermediate target, for instance chaperone proteins like Hsp70/90 that affect protein folding (see Discussion).

In agreement with the observed stimulation of DNA binding, treatment of mutant *p*53-carrying cells with PRIMA-1 resulted in the activation of the p53 targets MDM-2, p21 and PUMA. In contrast, PRIMA-1 treatment of cells carrying wild-type *p*53 did not result in p53 stabilisation or induction of p53 target genes. Restoration of wild-type p53 conformation by PRIMA-1 was associated with a reduction in total p53 levels. This is consistent with the notion that induction of MDM-2 upon mutant p53 reactivation promotes proteosomal degradation of p53. The importance of p53's transcriptional transactivation activity for PRIMA-1-mediated mutant p53-dependent apoptosis was verified by examination of the His-175 22/23 mutant p53 protein which is transcription-deficient due to substitutions of the N-terminal residues Leu-22 and Trp-23. Human tumour cells expressing the His-175 22/23 mutant were resistant to PRIMA-1 [21].

PRIMA-1 has mutant p53-dependent anti-tumour effect in SCID mice carrying human tumour xenografts of Saos-2 (p53 null) and Saos-2 His-273. No apparent toxic effects of PRIMA-1 treatment were observed [21]. As a continuation of these *in vivo* studies, it will be important to examine the effect of PRIMA-1 on spontaneously arising tumours in mutant p53 transgenic or knock in mice. More work is clearly also needed to elucidate the mechanism by which PRIMA-1 restores mutant p53, its specificity in various *in vivo* models, and the possibility of combining PRIMA-1 treatment with already established anti-cancer drugs or radiotherapy. Furthermore, screening of structural analogues will hopefully identify even more potent PRIMA-1 analogues for clinical trials.

PRIMA-1-induced mutant p53-dependent growth suppression was confirmed by an analysis of the available information in the National Cancer Institute database (http://dtp.nci.nih.gov), which has information on a large number of compounds, including many known anti-cancer drugs, and their effect on 60 human tumour cell lines (see above). Interestingly, the growth suppression effect of PRIMA-1 was proportional to cellular levels of mutant p53, but no correlation was found with levels of wild-type p53 or with cell proliferation rates [23]. PRIMA-1 was one out of two substances among the 2000 compounds in the Diversity set that showed consistent specificity for mutant p53-expressing cell lines in the database. In contrast, such clinically used anti-cancer drugs as cisplatin, 5-fluorouracil, methotrexate and doxorubicin preferentially targeted tumour cell lines carrying wild-type *p*53. Only paclitaxel showed some preference for cell lines expressing mutant p53 [23], in agreement with a previous study [24].

8. Discussion

Reactivation of mutant p53 in tumours has emerged as an attractive strategy for novel tumour therapies. Its allure lies in the fact that around 50% of human tumours carry mutant *p*53 and that such tumours often show increased resistance to conventional chemotherapy and radiotherapy. Novel drugs that target mutant *p*53-carrying tumours are thus urgently needed. Moreover, mutant p53 reactivation relies on the significantly elevated levels of mutant p53 proteins in most tumour cells compared with normal cells, providing the desired tumour cell specificity. Finally, it is essential that p53 reactivation in tumour cells will trigger apoptosis rather than cell cycle arrest, as the therapeutic goal is to kill the tumour cells, not just halt their growth.

The challenge of rescuing mutant p53 in human tumours seems formidable. A wide spectrum of tumour-associated *p53* mutations gives rise to a corresponding range of amino acid substitutions in the p53 protein. Some *p53* mutations may have no or little effect on protein folding, whereas others will disrupt local structure or cause denaturation [25]. Thus, the target may be viewed as not one protein, but many. Nonetheless, studies have indicated that reactivation of multiple mutant forms of p53 by small molecules can be achieved. Short synthetic peptides derived from p53's C-terminal tail were able to stimulate transcriptional transactivation by mutant p53 and also induce mutant p53-dependent apoptosis in human tumour cells. More recent screening of chemical libraries, using either protein *in vitro* assays or cellular assays, have yielded small molecules that restore native conformation and/or normal function to mutant p53, and prevent the outgrowth of human tumour xenografts *in vivo*.

How could small molecules such as CDB3, CP-31398 and PRIMA-1 restore wild-type conformation and tumour suppressor function to mutant p53? And how can a wide range of mutant p53 proteins be reactivated, as shown at least for PRIMA-1? The native state of a protein is the most thermodynamically stable conformation of the polypeptide chain under physiological conditions [26,27]. Mutations in p53 can probably affect thermodynamic stability of the protein fold by shifting the equilibrium towards a denatured state. A molecule with preferential binding to the native fold will most likely steer this equilibrium back towards the folded conformation by mass action, resulting in mutant p53 reactivation [25]. Thus, binding to the native conformation of p53 may be sufficient for mutant p53 reactivation. This may explain why a single small molecule can rescue a diversity of mutant p53 proteins.

The possibility that the identified mutant p53-reactivating compounds act through indirect mechanisms is equally plausible. Indeed, NMR analysis failed to reveal any direct physical interaction between CP-31398 and p53 [13], and a direct interaction between PRIMA-1 and p53 has not yet been demonstrated. Further studies using NMR and crystallography will be required in order to elucidate whether PRIMA-1 binds p53.

If a mutant p53-reactivating compound does not interact directly with the protein, how does it achieve its effect? One possibility is that the compound affects cellular chaperones, e.g. Hsp90, resulting in the refolding of mutant p53 and transactivation of p53 target genes (Fig. 2). Another possibility is that the compound blocks complex formation between mutant p53 and p73, leading to the release of active p73 that triggers proapoptotic target genes. Furthermore, it is conceivable that mutant p53 expression renders tumour cells more sensitive to the apoptosis-inducing effect of a small compound in various ways. For instance, mutant p53 could illegitimately transactivate specific genes whose protein products are targeted by the compound or somehow increase sensitivity to the compound. Global analyses of gene and protein expression patterns by

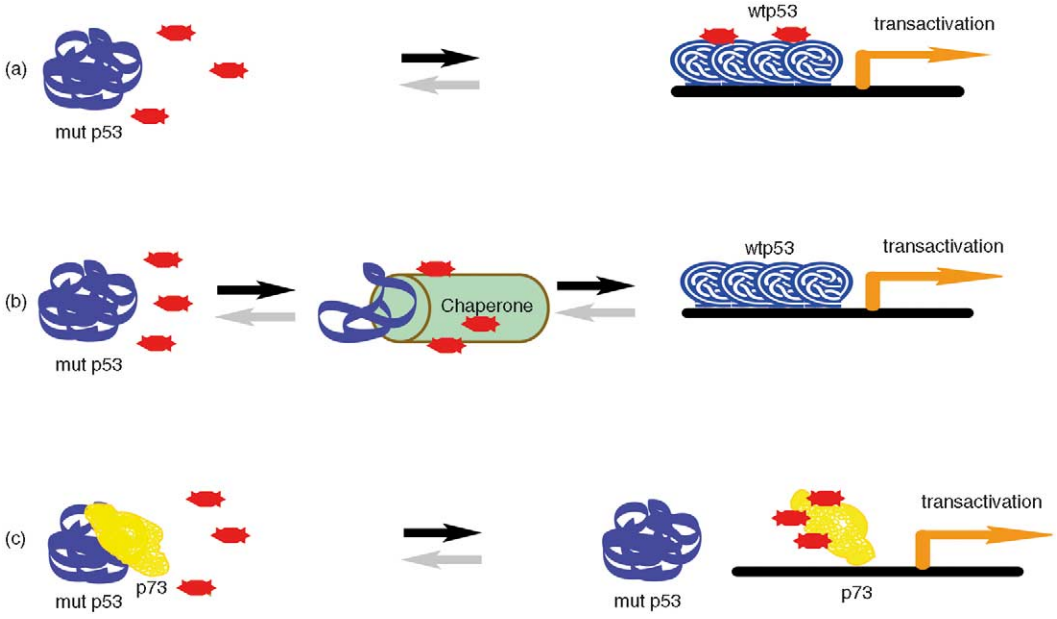

Fig. 2. Possible molecular mechanisms for mutant p53 reactivation by small molecules, leading to transactivation of growth-inhibiting and pro-apoptotic target genes. (a) Direct binding to mutant (mut) p53 and refolding. (b) Refolding of mutant (mut) p53 via a chaperone protein. (c) Disruption of mutant (mut) p53-p73 complexing, causing p73-dependent transactivation of target genes. mut p53: unfolded mutant p53; wtp53: mutant p53 refolded into the wild-type conformation.

DNA microarrays and proteomics techniques should clarify how mutant p53-reactivating drugs affect cells, and hopefully provide clues as to the molecular mechanisms of mutant p53 reactivation.

Development of resistance to therapy through clonal selection is a fundamental problem in all anti-cancer strategies. In the case of mutant p53 reactivation, loss of mutant p53 expression would be an obvious cause of resistance. However, gain-of-function activities of mutant p53 may contribute to the malignant phenotype, which would generate a selection pressure for maintaining mutant p53. Therefore, resistance via loss of mutant p53 expression could be less common than predicted if *p*53 mutation only served to inactivate wild-type p53 function. Nonetheless, the combination of mutant p53-reactivating drugs with conventional anti-cancer drugs and other therapeutic modalities will almost certainly be the most effective therapy regimen in the future.

In conclusion, several small molecules that are able to reactivate mutant p53 proteins have been identified. We need to learn more about their molecular mechanisms of action, optimise their structures, and test them in clinical trials. Mutant p53 reactivation by small molecules is a rapidly evolving field of translational cancer research with obvious potential for the generation of more efficient and specific anti-cancer drugs.

Acknowledgements

We thank the Swedish Cancer Society, Ingabritt & Arne Lundberg Research Foundation, the Cancer Society of Stockholm, Gustaf V Jubilee Fund, the Swedish Research Council (VR) and the Karolinska Institute for their generous support. V.N.J.B. was supported by a postdoctoral fellowship from the Swedish Cancer Society.

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