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## Perspective

### Drug-tolerant persister cells in cancer: the cutting edges and future directions

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## **Abstract**

Drug-tolerant persister (DTP) cell populations were originally discovered in antibiotic-resistant bacterial biofilms. Similar populations with comparable features have since been identified among cancer cells and have been linked with treatment resistance that lacks an underlying genomic alteration. While progresses in the past ten years have improved our understanding of the biological roles of DTP cells in cancer, clinical knowledge of the role of these cells in treatment resistance remains limited. Nonetheless, targeting this population is anticipated to provide new treatment opportunities. In this Perspective, we aim to provide a clear definition of the DTP phenotype, discuss the underlying characteristics of these cells, their biomarkers and vulnerabilities, and encourage further research on DTP cells that might improve our understanding and enable the development of more-effective anticancer therapies.

## [H1] Introduction

The introduction of effective systemic therapies such as chemotherapy, endocrine therapy, targeted therapy or immunotherapy, available in various clinical settings including locally advanced disease, adjuvant or neoadjuvant therapy<sup>1</sup>, has dramatically improved the clinical outcomes of patients with cancer, even those presenting with metastatic and/or unresectable lesions. For example, the third-generation EGFR inhibitor, osimertinib, has demonstrated superior efficacy to earlier-generation agents with a median progression-free survival (PFS) of 18.9 months and median overall survival (OS) of 38.6 months in the phase III FLAURA trial<sup>2</sup>. However, despite these major therapeutic advances and an improved understanding of the genetic determinants, resistance to EGFR inhibitors inevitably occurs, resulting in disease progression<sup>3</sup>. Similar challenges can be observed in patients with resected stage III *BRAF*-mutant melanoma, in whom relapse-free survival (RFS) is improved with adjuvant dabrafenib plus trametinib, but remains unsatisfactory with a 1-year RFS of 88%, declining to 52% at 5 years<sup>4, 5</sup>. Comparable clinical outcomes have been observed in patients receiving chemotherapy, such as those with acute myeloid leukaemia (AML), in which 70–80% of patients deemed fit for induction chemotherapy enter complete remission, yet the majority will eventually have disease relapse, especially those who are unable to undergo haematopoietic stem-cell transplantation<sup>6</sup>. Among patients with extensive-stage small-cell lung carcinoma (SCLC), which is often initially chemosensitive, response rates of 60–80% with complete response rates of 15% have been reported following treatment with a platinum-containing agent plus etoposide<sup>7</sup>. However, almost all patients will have disease progression, with a median PFS duration of 6 months and median OS ranging from 8–13 months<sup>8</sup>. Hence, standard-of-care (SOC) therapies for many advanced-stage cancers are rarely curative, and acquired resistance remains an unresolved clinical challenge, often despite favourable initial responses<sup>9</sup>.

Despite the consensus that disease relapse originates from minimal residual disease (MRD)<sup>10</sup>, an ongoing debate exists regarding various biological concepts such as tumour cell dormancy, cancer stem cells (CSCs), persistence, as well as the associations of these various phenomena with disease recurrence and therapeutic resistance in specific clinical scenarios<sup>11-13</sup>. For example, dormant tumour cells might exist in a reversible quiescent state prior to administration of local therapies, resulting in a long delay between successful treatment of primary tumours and metastatic relapse at secondary sites<sup>14, 15</sup>. On the other hand, CSCs are characterized by their ability to maintain an asymmetric self-renewal cellular state, expressing exclusive ‘stemness’ markers and thus occupying the apex of the differentiation hierarchy.

These characteristics result in CSC's potential for immune evasion and treatment resistance<sup>16, 17</sup>. The selective pressures imposed by systemic therapies might eliminate non-CSCs while sparing a population of intrinsically resistant CSCs, leading to an enrichment of CSCs in the MRD<sup>13</sup>. Both dormant tumour cells and CSCs typically exist as either single cells or small clusters of up to 20 cells that are highly dependent on their microenvironmental niche, in which their division might be suppressed for varying lengths of time by local immunosurveillance. These cells might then re-enter the cell cycle or undergo differentiation several years or even decades after the completion of curative-intent therapies<sup>18, 19</sup>. Initially characterized *in vitro* as a subpopulation of *EGFR*-mutant NSCLC-derived cancer cells that survive initial targeted therapy, drug-tolerant persister (DTP) cells are seen following continuous treatment with anti-cancer agents and have a reversible and heterogeneous phenotype that is not driven by *de novo* genomic alterations<sup>20</sup>. In contrast to dormant tumour cells or CSCs, DTP cells can re-enter a proliferative state and subsequently acquire stable resistance mutations in the presence of continuous treatment, thus serving as a reservoir for acquired resistance to systemic anticancer therapies<sup>21, 22</sup>.

From a clinical perspective, our understanding of DTP cells is often conceptually perplexing when compared to dormant tumour cells or CSCs. The coexistence of all of these scenarios in patients remains an open question, depending on residual disease after the type of local therapy and completion of adjuvant therapy, or during ongoing therapy. Therefore, understanding the various cellular states that might or might not reflect the full spectrum of cancer persistence, and a precise definition of the DTP phenotype are major research questions that will need to be addressed in order to advance this emerging field towards clinical applications. In this context, we propose a clear definition of the DTP phenotype and discuss various advances, as well as the opportunities and challenges associated with interventions targeting these resilient survivor cells<sup>9</sup>.

## **[H1] An updated view of DTPs**

### **[H2] An old concept made new**

The concept of drug-tolerant persistence was originally borrowed from a report of bacterial persister cells, first described by Joseph Bigger in 1944 after he had discovered a culture of *Staphylococcus aureus* that could not be sterilized with high doses of penicillin. Bigger hypothesized that a subpopulation of bacterial cells was able to survive exposure to bactericidal antibiotics owing to their lack of growth rather than any heritable mechanism of resistance<sup>23</sup>. Several mechanisms that might be responsible for this bacterial persister

phenotype have been described in studies of microorganism biofilms<sup>24, 25</sup>; however, specific interventions targeting these populations have rarely been tested in clinical trials. This lack of targeted clinical interventions most probably reflects the difficulties of identifying such cells in diverse populations of microorganisms and thus lead to the role of bacterial persisters in antibiotic resistance being largely overlooked<sup>24</sup>. More than 60 years after the discovery of bacterial persisters, research by Jeff Settleman's group showed that persistent cells with a reversible phenotype that was not determined by any underlying genetic alteration could also be observed in populations of NSCLC cells in vivo in response to anticancer therapies<sup>20</sup>. Similar to what Bigger had observed in bacteria, DTPs can repopulate drug-sensitive cellular progenies following a 'drug-holiday' without harbouring any known resistance-associated secondary mutations<sup>21</sup>. From the viewpoint of persistence dynamics, bacterial persisters and DTP cancer cells have similar cell death dynamics<sup>26</sup>, displaying classical biphasic kinetics after initial exposure to antibiotics or chemotherapies (BOX 1). Evidence supporting the existence of DTP cells has been reported in studies of various different cancer types, including but not limited to melanoma<sup>27, 28</sup>, basal cell carcinoma<sup>29</sup>, colorectal cancer (CRC)<sup>30-32</sup>, breast cancer<sup>33-35</sup>, pancreatic ductal adenocarcinoma<sup>36</sup>, glioblastoma<sup>37</sup> and AML<sup>38</sup>.

## **[H2] Defining drug-tolerant persistence**

The DTP phenotype is a temporary state of tolerance acquired by a small subset of tumour cells in response to initial systemic anticancer treatments that enables residual tumour cells to survive until more-permanent mechanisms of resistance can be established (BOX 2). When defining DTPs, three fundamental factors should be considered: interaction, stochasticity and evolvability (Fig. 1). Strictly speaking, DTPs are generated through specific interactions with the administered drug in a given environment and do not solely arise from the intrinsic properties of specific cells. An appropriate analogy is that of a sieve, through which objects can pass based on their size, although the separation occurs only through the rate at which objects pass through the sieve. In the context of Darwinian framework, we propose that the adaptations of individual cancer cells, which are defined by the specific response of each cancer cell during a certain interaction, have different implications. With this framework in mind, considering the role of specific treatments when discussing DTPs and their reversible phenotype is essential. For example, where MRD-driven disease relapse occurs after local therapy (such as surgery) in the absence of systemic adjuvant therapy, this more likely reflects the presence of disseminated dormant tumour cells or CSCs rather than DTP cells. In certain scenarios, such as MRD that survives adjuvant therapy or an incomplete response to systemic

therapy in the context of advanced-stage disease, distinguishing between the effects of DTPs, dormant tumour cells, CSCs or primary resistant tumour subclones can be challenging<sup>39</sup>. Therefore, the context-specific interactions of a treatment with cancer cells must be included as a fundamental component of the DTP phenotype; however, this inclusion introduces two levels of variability. First, all cancer cells in an isogenic population have the potential to stochastically acquire and yield a tolerant phenotype, albeit usually at a low prevalence. Data from single-cell barcoding experiments indicate that varying proportions of fast-cycling and slow-cycling cells exist within each barcoded DTP lineage, suggesting that entry into the DTP state is a stochastic process<sup>40</sup>. Furthermore, a fraction of untreated cells might exist in a ‘pre-DTP’ primed state at any given time, adding a layer of variation to the sieve analogy: objects randomly change their sizes before the interaction with a specific sieve. For example, pre-DTP cells expressing neuropeptide Y receptor Y1 (NRY1R) or ATP-binding cassette subfamily C member 5 (ABCC5) in an ex vivo population of human HER2<sup>+</sup>/ER<sup>-</sup> breast cancer cells, are predisposed to becoming de facto DTPs upon exposure to HER2 tyrosine-kinase inhibitors<sup>41</sup>. Similarly, in cells derived from patients with *BRAF*<sup>V600E</sup>-mutant melanoma, a subset of cancer cells expressing histone demethylase KDM5B can enter a DTP state on exposure to vemurafenib. However, this primed pre-DTP state reflects a stable equilibrium, with *KDM5B*-positive and *KDM5B*-negative melanoma cells stochastically interconverting. Thus, the emergence of melanoma DTP cells seems to be dependent on interactions between the cancer cells and a BRAF inhibitor<sup>42, 43</sup>.

Another layer of stochasticity also arises from interactions between cancer cells and the specific treatment, leading to phenotypic heterogeneity among DTP cells. Depending on the specific cancer types and treatment strategies, DTP cells might be identified based on the detection of one or more of several other features including incomplete apoptosis<sup>44</sup>, ferroptosis<sup>45</sup>, pyroptosis<sup>46</sup>, genomic instability<sup>26</sup>, quiescence, diapause-like status<sup>47</sup> and phenotype switching. Within DTP populations, individual tolerant cells might adopt various transcriptional programmes, metabolic states and/or proliferative capacities, even though they are interacting with the same treatment. Data from an elegant in vitro analysis demonstrate that proliferating DTP cells require a balanced intracellular redox state associated with increased fatty acid oxidation, whereas non-proliferating DTP cells are characterized by upregulation of Notch signalling and type I interferon-related genes in cell lines derived from *EGFR*-mutant NSCLC, *HER2*-positive breast cancer or *BRAF*-mutant melanoma<sup>48</sup>. Moreover, biological processes or pathways contributing to drug tolerance are seemingly distributed across various DTP cell subpopulations rather than co-existing in individual DTP cells. This

variability has been demonstrated in vitro using *EGFR*-mutant NSCLC-derived cell lines exposed to erlotinib, in which prolonged exposure (for 11 days) led to the emergence of individual clusters of erlotinib-tolerant cells and larger DTP cell subpopulations with distinctive molecular phenotypes, such as altered cholesterol metabolism, EMT or epigenetic deregulation<sup>49</sup>. Thus, the landscape of cellular plasticity that DTP cells can potentially exploit upon exposure to anticancer therapies has certain biological similarities with disseminated tumour cells, or those of a dormant or CSC phenotype.

The concept of drug-tolerant persistence arises to address the clinical paradox posed by patients who respond to reintroduction of the same therapy after a drug holiday, despite previous disease progression on the same agent<sup>20</sup>. Evidence also suggests that continuing therapy beyond disease relapse can still provide tumour control in certain circumstances. In a few specific cases, including some CRCs<sup>50</sup>, SCLC<sup>51</sup> or ovarian cancer<sup>52</sup>, drug rechallenge has led to more flexible definition of acquired drug resistance, in which clinical relapses may be non-genetic or partially genetic if a response to retreatment is observed. For example, in a clinical trial by using *EGFR*-targeting monoclonal antibodies for treating colorectal cancers, rechallenge has been tested based on absence of resistant genetic alterations in circulating tumour DNA (ctDNA)<sup>53</sup>. However, the scenarios in which rechallenge might be feasible are likely biologically and clinically distinct and therefore highly context-dependent. Nonetheless, the anticancer activity that can be achieved in some patients indicates that many seemingly resistant tumours were either not truly resistant at initial disease progression or that the resistant phenotype arising following an initial response was transient. On the basis of data on tumour dynamics, tumour heterogeneity and response to therapy, the capacity of DTP cells to evolve upon exposure to anticancer therapies is an important feature that distinguishes such cells from dormant tumour cells or CSCs. During a drug holiday or in a rechallenge scenario, DTP cells can expand and generate progenies that are sensitive to the same treatment; nonetheless, such cells can also revive their DTP phenotype and/or mutate to produce genetically altered, irreversible treatment-resistant clones upon continuation of treatment<sup>20, 47</sup>. Consequently, different DTP states or behaviours probably exist depending on the disease type and treatment received, and the DTP population rarely comprises cells of a single monolithic phenotype. Thus, careful definition of DTP characteristics within their specific contexts, including the disease setting, clinical management approach and underlying biology of the tissues involved, is essential when attempting to investigate this effect. The defining biological characteristics of DTP cells in different contexts have been described extensively elsewhere<sup>9, 54-56</sup>.



## [H2] Appearances can be deceiving

As mentioned previously, DTP cells are phenotypically heterogeneous, typically manifesting as either slowly proliferating or entirely quiescent cells in a histology-dependent manner<sup>37</sup>. This slow-growing or quiescent phenotype often leads to a debate as to whether DTP cells are in fact synonymous with dormant tumour cells. The latter are a population of malignant cells that have exclusively undergone G0–G1 cell cycle arrest which is a prerequisite for dormancy<sup>57</sup>. However, DTP cells adopt cell states either arrested at this cell cycle phase or slow-proliferative in a cancer type-dependent manner. A pan-cancer analysis of the extent of tumour cell dormancy based on TCGA data revealed a spectrum of dormancy scores<sup>58, 59</sup> (reflecting the expression of 139 dormancy-related genes) among different cancer types at baseline, with adrenocortical carcinoma and glioblastoma having the highest scores and testicular germ cell tumours and thymomas having the lowest<sup>59</sup>. Notably, residual DTP cells in patient-derived xenograft models of CRC largely maintain low levels of Ki-67 expression following exposure to standard-of-care 5-fluorouracil (5-FU), leucovorin and oxaliplatin (FOLFOX)<sup>31, 47</sup>, suggesting that such cells tend to retain a proliferative phenotype, which is consistent with the low levels of tumour cell dormancy reported in this cancer type<sup>59</sup>. An analysis of patient-derived CRC organoid responses to chemotherapy demonstrated that 50% of DTP cells defined by expression of the RNA binding protein MEX3A reside in the G0, and 38% in the G1 phase of the cell cycle<sup>32</sup>. However, these DTP cells were derived from cancer cells expressing LGR5, a known marker of stem cells in the small intestine and colon<sup>60, 61</sup>. This observation implies that MEX3A<sup>+</sup> DTP cells are derived from a pre-existing population of progenitor cells<sup>32</sup>. Melanomas included in TCGA have moderate tumour dormancy scores, with cell cycle arrest observed only in cells with a starvation-like state<sup>28, 62</sup>. By contrast, barcoding study of glioblastoma DTP cells demonstrated a high level of dormancy<sup>63</sup>, which is consistent with the high prevalence of dormancy signatures observed in the glioblastoma TCGA dataset<sup>59</sup> and could be partially attributed to assembly of the dimerization partner, RB-like, E2F and multi-vulval class B (DREAM) complex mediated by DYRK1A kinase<sup>64</sup>. The DREAM complex mediates cell-cycle associated gene suppression during G0 and coordinates periodic gene expression during G1/S and G2/M phases<sup>65</sup>, whose perturbation shifts the balance between quiescence and proliferation. This has been widely exploited as a treatment target for intervening tumour cell dormancy<sup>66</sup>.

Unlike the exclusively quiescent phenotype of dormant tumour cells, a subset of DTP cells is primed to enter the cell cycle and thus facilitate disease relapse upon exposure to

treatment. For example, an investigation using a high-complexity tracing system to trace the clonal origins of individual cells within an *EGFR*-mutant NSCLC cell line while simultaneously tracking proliferative and transcriptional states demonstrated that at least 13% of barcoded DTP cells formed larger colonies, suggestive of a stronger proliferative phenotype, and that as many as 6% of cells exhibited some extent of DTP-like phenotype<sup>48</sup>. Using longitudinal single-cell RNA sequencing of DTP cells of specific lineages, these investigators showed that proliferative DTP cells require a balanced intracellular redox state, such as that associated with increased fatty acid oxidation<sup>48, 67</sup>. Redox homeostasis might be regulated by NPC1L1 through the promotion of vitamin E uptake, as demonstrated in DTP cells generated from ABCB1<sup>+</sup> breast cancer cells exposed to paclitaxel or adriamycin in combination with the ABCB1 inhibitor verapamil<sup>68</sup>. Non-proliferating DTP cells are characterized by upregulation of pathways associated with Notch signalling and genes involved in type I interferon signalling<sup>48</sup>. In melanoma, cycling DTPs might at least rely on stochastic bursts of oncogenic signalling for proliferation: for example, 5% of *BRAF*<sup>V600E</sup>-mutant melanoma-derived DTP cells have sporadic ERK phosphorylation pulses of lasting 60–90 minutes when exposed to BRAF inhibitors in vitro, thus generating slowly-dividing DTP cells<sup>69</sup>.

## **[H2] Primed DTP cells might have memory**

Data published in 2022<sup>70</sup> described the exploration of DTP cancer cell dynamics using a modified Luria–Delbrück fluctuation-test framework<sup>71</sup> in human CRC cells exposed to the anti-EGFR antibody cetuximab in vitro. These investigators used a mathematical model to determine the pre-existence of a steady fraction ( $f_0$ ) of DTP cells and found that the abundance distribution of such cells follows a Poisson distribution, thus supporting a drug-induced scenario. The authors found that  $f_0 = 0$  provided the best level of agreement between their model and experimental data, although they also could not rule out the possibility of a few DTP cells existing before exposure to cetuximab. Data from a different study used similar methods to identify an extremely rare population of DTP cells among human *BRAF*<sup>V600E</sup>-mutated melanoma cells that are present prior to exposure to BRAF inhibitors and are able to persist in vitro in the presence of these agents<sup>72</sup>. Other data published in 2022 indicate that randomly proliferating HER2<sup>+</sup> breast cancer cell lines stochastically maintain a population of cells, referred to as ‘pre-DTP’ cells, which have a G<sub>0</sub>-like gene expression signature with differential expression of a subset of genes that are also expressed in DTPs, before treatment with the anti-HER2 antibody lapatinib<sup>41</sup>. Moreover, these pre-DTP cells had gene expression

patterns similar to those of diapause-like and senescence-like transcriptional programmes, which have also been observed in DTP cells originating from patient-derived xenograft (PDX) models of CRC<sup>31</sup> and patient-derived organoid or xenograft models of breast or prostate cancer<sup>47</sup>. Bulk RNA sequencing of tumour tissues obtained from patients with HER2<sup>+</sup> breast cancer in the NeoALTTO trial<sup>73</sup> revealed higher pre-DTP gene signature scores among patients who did not have a pathological complete response (pCR) to neoadjuvant lapatinib compared to those with a pCR. These findings suggest that, at least in HER2<sup>+</sup> breast cancer, a stable population of DTP cells might not exist before treatment; rather, that tumour cells stochastically transition through G<sub>0</sub> to enter a primed pre-DTP state, and that these cells preferentially proceed to become bona fide DTP cells upon exposure to HER2-targeted therapies. These observations are consistent with the drug-tolerant cellular state observed in both PDX models of and in patients with triple-negative breast cancer (TNBC) receiving adriamycin and cyclophosphamide in the neoadjuvant setting<sup>34</sup>. These researchers found that all cancer cell subclones are equally capable of adopting a DTP state characterized by a distinct stromal component and cancer cell pleomorphism<sup>34</sup>.

Interestingly, intermittent application of antibiotics to a population of bacteria can give rise to isolated stable higher persister (*hip*)-mutant cells, and at least one gain-of-function allele, *hipA7*, has been shown to enhance bacterial persistence up to 1,000-fold in the presence of antibiotics<sup>74</sup>. Although genomic alterations that increase the frequency of DTP cells have thus far not been identified in cancers<sup>31</sup>, cancer cells have been shown to have a highly stable chance to persist in response to EGFR inhibitors that is determined by the phosphorylation status of insulin receptor substrate 1 (IRS1) in cell line and PDX models<sup>75</sup>. Investigators report that this mechanism is probably treatment specific. Data from earlier studies indicate that phosphorylation events can indeed confer long-term memory, as shown in the context of neurological long-term potentiation<sup>76</sup>. The chance of DTP cells persisting after a drug holiday or alternative treatment, and resulting in resistance upon drug rechallenge, might therefore have a heritable component<sup>75</sup>. These observations raise the intriguing question as to whether the previously described pre-DTP state could confer long-term memory leading to cancer persistence. Exposure of melanoma cells to hypoxia or nutrient starvation can lead to a drug-tolerant cellular state that confers resistance to both BRAF inhibitors and platinum-based chemotherapy<sup>77</sup>. These stress response-primed DTP cells underwent widespread chromatin remodelling accompanied by loss of H3K4me3 and H3K27me3 marks and gain of H3K9me3, which has also been observed in DTP cells derived from an *EGFR*-mutant NSCLC cell line exposed to erlotinib<sup>78</sup> and in PDX models of TNBC exposed to 5-FU<sup>35</sup>.

Whether epigenetic alterations in DTP cells are preserved as a heritable memory in cells that revert to a drug-sensitive cellular state, thus providing an evolutionary pathway that enables an increase in the frequency of persister cells upon drug rechallenge, remains to be investigated. This possibility has been explored in *Escherichia coli* DTP cells, which follow a ‘last in, first out’ rule at a single-cell level, whereby cells that are the last to shut down their metabolism when entering a persistent state are also the first to become reactivated in response to nutrients<sup>79</sup>. These observations suggest that the physiological state of the offspring reflects the past state of its ancestor<sup>80</sup>, which could explain the often improved survival of cancer cells upon continued treatment.

## **[H2] To die or not to die**

At the population level, variations in the chance of persistence have an important role in determining the fraction of cancer cells eradicated by therapy and the frequency of DTP cells over time<sup>75</sup>. However, at the cellular level, the emergence of DTPs following anticancer treatment is primarily determined by the balance between cell survival and death. The extent to which cell-intrinsic death programmes become activated dictates the ‘elbow’ – the percentage of cells that die immediately following initial treatment – of the dying kinetic curve observed in DTP cells (BOX 1). In this regard, developing a population of DTP cells due to failed apoptosis results in a worse clinical outcome than cell death, as demonstrated by the existence of cells exposed to TRAIL that undergo only limited caspase activation and are able to survive despite mitochondrial outer membrane permeabilization (MOMP) and compromised X-linked inhibitor of apoptosis proteins (XIAP) expression<sup>81,82</sup>. Previous research indicates that the P-glycoprotein-expressing K562/ADR cell line, a model of resistance to doxorubicin, has cytosolic and mitochondrial holocytochrome c activity that does not lead to cell death. Notably, mitochondria from this cell line spontaneously release holocytochrome c, indicating altered MOMP processes that could be blocked by BCL-2 overexpression<sup>83</sup>. Data published in 2022 suggest that incomplete MOMP and holocytochrome c release might have a key role in the generation of DTP cells in cell line and mouse xenograft models of NSCLC exposed to BH3 mimetics<sup>44</sup>. This sublethal release of cytochrome c into the cytoplasm activates heme-regulated inhibitor kinase, leading to eIF2 $\alpha$  phosphorylation and subsequent ATF4-dependent translational reprogramming, with an analysis of patient samples suggesting activation of such signalling pathways in samples obtained from patients with several solid tumour types. This reprogramming leads to subsequent metabolic rewiring, cell-cycle inhibition and EMT, potentially facilitating the

ability of DTP cells to evade anticancer therapies. However, generalizing the mechanism of DTP cell generation based on these data from experimental studies of the downstream effects of failed apoptosis to other anti-cancer treatments is currently inadvisable due to the development of DTP cells is likely to be treatment dependent as mentioned above. Notably, anti-PD-1 antibody-induced CD8<sup>+</sup> T cell-dependent cancer cell death has been shown to engage ferroptosis, an alternative form of cell death, and expression of ferroptosis-related genes in TCGA data from patients with melanoma is associated with improved survival<sup>84</sup>. Thus far, limited evidence exists supporting a DTP effect following immunotherapy, including immune-checkpoint inhibition or adoptive T cell transfer (ACT). Using single-cell sequencing of mouse-derived organotypic tumour spheroids, investigators identified a discrete subpopulation of persister cells that can resist CD8<sup>+</sup> T cell-mediated cell death following exposure to an anti-PD-1 antibody. These residual cells express hallmark gene sets of EMT with the expression of known EMT promoting factors (i.e. *Snail*, *Lif*, *Socs3* and *IL-6*) and are supported by expression of IL-6 mediated baculoviral IAP repeat-containing protein 2/3 (BIRC2/3) signalling axis, whose inhibition in combination with anti-PD-1 antibody leads to cell death<sup>85</sup>. In a study involving a mouse model of squamous cell carcinoma harbouring CSCs, ACT led to the emergence of a population of resistant cells expressing CD80, which enables CTLA4 engagement and direct suppression of CD8<sup>+</sup> T cell activity<sup>86</sup>. However, this study was not performed in the context of cancer persistence, further investigations will be required to understand if these ACT tolerant cells are induced DTP cells sharing CSC-like phenotype or pre-existed CSCs. Nevertheless, evidence exists that immunosuppressive proteins can indeed be upregulated on the surfaces of DTP cells. For example, CD70 upregulation has been reported in a study of osimertinib-resistant *EGFR*-mutant NSCLC cells derived from a cell line, in which CD70 upregulation promotes DTP cell survival by triggering reactivation of the PI3K signalling pathway. CD70 upregulation is shown to be an early event in the evolution of resistance and exclusively expressed in residual drug tolerant persister cells in this model<sup>87</sup>. CD70 is known to interact with CD27 expressed on the surfaces of naive and memory T cells<sup>88</sup>, the investigators demonstrated that anti-CD70 antibody drug conjugates and CD70-targeting chimeric antigen receptor T cells or NK cells can potentially eliminate CD70 expressing DTPs<sup>87</sup>. These data therefore open an intriguing area for future research.

## **[H2] All roads lead to resistance**

By studying the pharmacodynamics of bacterial persistence, Johnson and Levin proposed the ‘persistence as stuff happens’ (PaSH) hypothesis, suggesting that bacterial persistence is a consequence of various types of replication errors, such as mutations<sup>89</sup>. Similarly, DTP cancer cells can have various phenotypes that enable them to survive following exposure to systemic therapies, thus contributing to the ability of DTPs to transition to more-permanent acquired resistance<sup>90</sup>. An analysis of samples from patients with microsatellite-stable CRC demonstrates the downregulation of genes involved in mismatch repair (MMR) and homologous recombination with concomitant upregulation of error-prone DNA polymerases, such as POL $\kappa$  and POL $\iota$ , in PDX models exposed to cetuximab with or without dabrafenib and in patients receiving cetuximab plus chemotherapy<sup>30</sup>. The increased expression and activity of these low-fidelity DNA polymerases results in the emergence of DTP cells with mutation rates 7–50 times higher than those of the treatment-naive parental CRC cell population, despite slower replication during drug exposure<sup>70</sup>. Similarly, in *EGFR*-mutant NSCLC cells, the upregulation of error-prone DNA polymerases seen in response to EGFR inhibitors might be mediated by autocrine GAS6–AXL signalling<sup>91</sup>. Activated AXL kinase directly binds to the DNA polymerase co-organizer RAD18 and promotes its neddylation, thus facilitating error-prone translesion synthesis (TLS) that directly replicates DNA damages<sup>91, 92</sup>. In *EGFR*-mutant NSCLC cells, AXL overexpression enhances the survival of DTP cells and accelerates the emergence of *EGFR*<sup>T790M</sup>, an established resistance mutation, in cells exposed to erlotinib<sup>91, 93</sup>.

One question for future investigation relates to how cancer DTP cells can create DNA damage without undergoing cell death upon drug exposure. Schmitt et al. proposed that symbiotic interactions between tumour cells might promote cancer cell survival and DNA damage in human CRC<sup>94</sup>. Upon 5-FU exposure, ATP released from dying cancer cells can trigger P2RX4 activation, leading to elevated production of reactive oxygen species (ROS) and subsequent accumulation of DNA damages in neighbouring cancer cells<sup>94</sup>. This symbiotic interaction creates a novel dependency on mTOR signalling pathways and promote the survival of the neighbouring stressed cells upon chemotherapy treatment<sup>94</sup>. Whether similar accumulation of ROS-mediated DNA damages is applicable to DTP cells and the interaction between DTP and sensitive dying cells will need further investigations. Failed apoptosis might be another mechanism of DTP-induced DNA damage. For example, sublethal caspase activation, such as caspase 3 activation owing to incomplete MOMP, could trigger limited cleavage of caspase-activated DNases (CADs)<sup>95, 96</sup>, leading to CAD-dependent DNA damage and genomic instability<sup>97</sup>. Indeed, cells from a glioma cell line surviving in vitro exposure to

TRAIL accumulate DNA damage in a caspase-dependent and CAD-dependent manner<sup>98</sup>. Moreover, repeat exposure to sublethal stress can promote cellular transformation and tumorigenesis in a MOMP-dependent manner<sup>97</sup>.

### **[H1] Translating DTP cell detection from bench to bedside**

Similar to bacterial persister cells, a major challenge in evaluating therapeutic strategies involving DTP cells in clinical settings is the difficulty in detecting and/or monitoring this residual population. DTP cells are characterized as a rare, slowly dividing or quiescent subpopulation of cancer cells with altered dependencies on certain signalling pathways and/or other features including metabolic alterations, that might be constitutive or inducible<sup>9, 31, 47, 48</sup>. Thus determining their kinetics throughout the course of therapy is necessary (Fig. 1). Furthermore, DTP cell phenotypes can vary widely between different cancer histologies and in response to different treatments, making the identification of one or more universally applicable clinically relevant biomarkers for DTP cells challenging. To date, virtually all studies focusing on the role of these cells in cancer have involved in vitro or ex vivo models; therefore, no gold-standard clinical biomarkers have been established, and this remains an open and important research question that will need to be addressed.

We propose that the kinetics of DTP cancer cells during treatment can be determined clinically using metabolic imaging to quantify the fraction of residual cancer cell populations present at the maximal treatment response. DTP cells could be further evaluated by analysing sequentially obtained blood samples for circulating DTP cells (cDTPs) and DTP-derived circulating cell-free tumour DNA (ctDNA), depending on their functional and dynamical features. Nevertheless, the detection of a measurable PET–CT signals might be precluded by the small fraction of viable DTPs that are likely present in most tumours. In addition, due to their scarcity and lack of specific biomarkers for DTPs, current liquid biopsy-based technologies will need further refinement for detecting cDTPs as well as distinguishing them from other primary resistant residual tumour cells. Future comparative studies evaluating the minimum fractions of DTPs detectable using these various methodologies will be needed to define the sensitivity of each approach.

### **[H2] Clinical imaging of DTP cells**

<sup>18</sup>F-fluorodeoxyglucose (<sup>18</sup>F-FDG) PET–CT has been used to predict residual metabolic activity after radiotherapy in retrospective studies<sup>99-101</sup>, although this approach might not be effective for monitoring most DTP cells, as they do not necessarily depend on glycolysis<sup>67, 102</sup>.

DTPs from multiple cancer types have been found to rely on iron-dependent lipid peroxidases (Fig. 2a)<sup>103, 104</sup>, and iron and 2-ketoglutarate ( $\alpha$ -KG)-dependent demethylases, including DTP cells derived from NSCLC cell line models or xenograft models<sup>78</sup>, breast cancer cell lines and xenografts<sup>35</sup> and melanoma KDM5B-expressing xenograft model<sup>105</sup>. Therefore, gallium-68 (<sup>68</sup>Ga)-citrate, an Fe (III) biomimetic, might be a promising alternative radiotracer for DTP cell detection (Fig. 2a). <sup>68</sup>Ga-citrate has been shown to accumulate in mouse models of PDAC in a transferrin receptor-dependent manner<sup>106</sup>, and data from clinical studies indicate that <sup>68</sup>Ga-citrate PET-CT enables the detection of various cancer types, including grade III-IV gliomas<sup>107</sup>, prostate cancers<sup>108</sup> and hepatocellular carcinomas<sup>109</sup>. Certain lesions may be easier to take up more <sup>68</sup>Ga-citrate depending on their metabolic features<sup>110</sup>. However, it remains to be determined if the alternative probes would be sufficiently sensitive to permit DTP cell monitoring in the context of MRD. Other metabolic radiotracers such as <sup>11</sup>C-acetate, have been proposed as methods of evaluating tumours that rely more on fatty acid metabolism than glycolysis, with preliminary data available from patients with prostate cancer<sup>111</sup> or hepatocellular carcinoma<sup>112</sup>. DTP cells utilize a variety of forms of fatty acid metabolism in different cancer types, therefore dual imaging using <sup>11</sup>C-acetate and <sup>18</sup>F-FDG might be an interesting approach<sup>112</sup>.

Future clinical trials integrating the use of these alternative radiotracers with <sup>18</sup>F-FDG PET-CT will require the reconsideration of clinical end points, as conventional end points such as radiographic response, progression-free survival and OS are insufficient for measuring DTP cells. For example, if the presence of, or numbers of DTP cells do not correlate with OS or PFS, the presence of such cells would likely be dismissed as irrelevant. Otherwise, a key point of the prognostic value would be to determine the clinical relationship between MRD, in particular MRD involving DTPs and clinical outcomes. Therefore, the development of effective DTP cell-targeted metabolic radiotracers will be a possible route to enable quantification of the fraction of residual cancer cell populations present at the time of maximal treatment response, which we term 'persistent tumour burden' (PTB). Combining DTP-targeting metabolic radiotracers with <sup>18</sup>F-FDG PET-CT could provide a more-precise and earlier readout of residual tumour burden and facilitate the development of DTP cell-targeted therapeutics. Prospective clinical trials incorporating DTP-relevant end points, such as PTB, will be needed to evaluate the utility of these alternative radiotracers. Trials testing neoadjuvant therapies provide a valuable opportunity to test DTP-targeting metabolic radiotracers during the limited period before surgical resection. Furthermore, the availability of resection specimens implied by this approach will enable the upfront multiplexed analysis



of residual disease following an incomplete response to neoadjuvant treatment. In this aspiration, an ongoing clinical trial (*ClinicalTrials.gov ID: NCT04816838*) use single cell RNAseq to investigate DTP cells after neoadjuvant treatment of osimertinib in patients with resectable NSCLC with EGFR mutations. The same group has also registered a single arm, open label, phase II trial (*ClinicalTrials.gov ID: NCT05361564*) for patients with resectable NSCLC harbouring ALK fusions, cell-free biomarkers related to DTP cells will be explored in tumours preoperatively treated with Brigatinib.

## **[H2] Capturing circulating DTPs**

A radiological complete response implies a lack of detectable tumour at a macroscopic level<sup>113</sup>, which might not accurately represent the intrinsically heterogeneous tumour mass. To overcome this clinical challenge, longitudinal follow-up involving minimally invasive methods is therefore a more appropriate method of MRD detection and monitoring. Advances in the capture and detection of circulating tumour cells (CTCs), the quantity and properties of which provide a snapshot of tumour burden at a specific timepoint<sup>114, 115</sup>, offer a potential solution<sup>116, 117</sup>. However, current CTC capture techniques rely on either physical or biological properties, with the relevant physical properties, such as larger cell size, lowest level of deformability, altered cellular density and cell-surface electrical charge<sup>118</sup>, being insufficiently studied for the high-sensitivity detection of cDTP cells (Fig. 2b). An alternative approach involves immunoaffinity-based CTC isolation, either with or without cell selection based on imaging of specific biological features<sup>119</sup>. FDA-approved tests, such as CellSearch system<sup>TM</sup>, enable the detection and enumeration of CTCs in most solid tumours, for example, based on the expression of EpCAM, and cytokeratins 8, 18 and 19, these methods could detect CTCs in patients with metastatic breast cancer, prostate cancer or CRC<sup>120</sup>. Adapting these techniques to putative DTP-specific biomarkers, such as the fatty acid metabolism-associated lipid transporter CD36<sup>121-123</sup>, would aid in the capture and monitoring of cDTPs cells, which could enable the early detection of residual populations that are able to tolerate anticancer treatment (Fig. 2b). Another potential trait enabling cDTP cell detection could involve the integration of cell-surface markers associated with EMT, such as CD109<sup>124</sup>, into immunoaffinity-based cDTP isolation. EMT-associated markers could also be expressed by some CSCs, which may lead to difficulties for the cDTP isolation. CTC isolation may enable ex vivo test, such as sphere-forming assay<sup>125</sup>, for CSC detection. In addition, CSC-related biomarkers may be also possible to be tested to exclude circulating CSCs in this context<sup>126</sup>. Combining alternative radiotracers with longitudinal cDTP monitoring would probably

enhance the level of sensitivity at which PTB can be detected and monitored in clinical settings. DTP cells are a highly heterogeneous cancer cell subpopulation characterized by various different evolutionary states with varying levels of proliferation leading to subclassification as non-proliferative or minimally proliferative DTP cells versus proliferating DTP cells, which can also be referred to as drug-tolerant expanding persisters (DTEPs)<sup>20, 41</sup> or drug-tolerant proliferating persisters (DTPPs)<sup>127</sup>, that might reflect unique vulnerabilities and mechanisms of drug resistance. In such a context, determining the ratio of cDTP cells to CTCs could help to characterize the resistance trajectory. Moreover, whether all CTCs retain DTP-specific features remains unclear, especially if the CTCs are exposed to distinct stressors that confer a unique phenotype that might or might not be classified as a DTP-specific feature.

## **[H2] DTP-derived ctDNA**

In contrast to the complex technical requirements associated with CTC isolation, ctDNA has the advantage of being easier to detect in plasma. Improvements in the sensitivity of next-generation sequencing (NGS) and analytical error corrections, for instance to eliminate CHIP mutations, have considerably improved the sensitivity of ctDNA detection<sup>128</sup>. Clinical trials testing ctDNA-guided therapy have demonstrated that ctDNA levels are correlated with radiological features and have independent prognostic value in metastatic settings<sup>53, 129, 130</sup> or in the context of MRD<sup>131, 132</sup>. Current ctDNA detection and analysis methods mostly involve NGS-based analysis of selected genes potentially harbouring oncogenic alterations and/or analysis of DNA methylation, which is technically feasible but not widely used yet in clinics<sup>133</sup>. Epigenetic alterations are recognized as a hallmark of DTP cells<sup>9</sup>; therefore, integrating DTP-specific chromatin modifications into ctDNA detection and analysis protocols could provide an important method of tracking dynamic changes in the DTP cell population during treatment. Examples of such alterations include increased H3K9me3 methylation resulting in the suppression of long interspersed repeat 1 elements, which is required to maintain DTP cell survival in an *EGFR*-mutant NSCLC cell line exposed to erlotinib<sup>78</sup>. Elsewhere, DTP cells derived from a TNBC cell line featured with H3K27me3-mediated suppression of several persistence-associated genes, which can be reversed by EZH2 inhibition, leading to the development of a 5-FU-tolerant phenotype<sup>35</sup>. Analysis of DNA methylation and chromatin remodelling in ctDNA is becoming feasible, enabled by methods such as cell-free chromatin immunoprecipitation<sup>134</sup>, which could be adapted for the detection of DTP-specific alterations (Fig. 2b). As a method of non-invasive DTP cell detection and monitoring, ctDNA detection seems less technically demanding than use of

CTCs. Nonetheless, cDTPs have several potential advantages, such as enabling the detection of a small fraction of CTCs that have properties that are likely not representative of cancer cell population in general and monitoring the clonal evolution of their drug tolerance, and potentially serving as a platform for drug screening that would otherwise specifically target DTP cells<sup>135</sup>. DTP cells are a highly heterogeneous subset of cancer cells often with unique vulnerabilities and tolerances<sup>41, 127</sup>. Therefore, the monitoring of DTP cell subsets present in CTC populations could be combined with the specific detection of DTP cell-derived ctDNA with comparisons to ctDNA derived from the non-persistent population. Despite the promise of non-invasive DTP cell detection and monitoring strategies, sufficiently specific, sensitive and clinically applicable DTP cell detection assays still need to be developed and will require considerable research and development efforts.

## **[H1] The therapeutic landscape of DTP cells**

DTP cells are a subpopulation of cancer cells that emerge from a mostly treatment-sensitive population that are able to survive initial treatment, resulting in MRD that is usually not detectable with conventional clinical evaluations<sup>9, 93, 136</sup>. Over the past decade, a large body of data from in vitro and/or ex vivo studies have shed light on the biological mechanisms of DTP cell development and function<sup>9, 54</sup>. Nonetheless, the relevance of these cells to the conventional clinical definitions of response, stable disease and/or disease progression remain poorly understood<sup>137</sup>. Thus, determining whether an incomplete response reflects primary resistance, the presence of residual DTP cells or other mechanisms of acquired resistance is crucial for the development of therapeutic strategies that lead to deeper and more durable responses and avoid treatment failure. Most cancers are, to varying degrees, genomically unstable<sup>138</sup>, and resistance is largely thought to originate from the genomic heterogeneity present within the tumour or tumours before the initiation of systemic treatment<sup>139</sup>. However, the selection of treatment-resistant subclones harbouring pre-existing genomic alterations that confer drug resistance is probably not the only mechanism of resistance to anticancer therapies. Transient effects may provide initial persistence, allowing a small subpopulation of tumour cells to escape the fitness threshold and survive until some acquired secondary mutations emerge<sup>140</sup>, although the clinical evidences are not established yet. A wide variety of mechanisms that contribute to the persistence of cancer cells have been reported in preclinical models<sup>9</sup>, including epigenetic, transcriptional and translational processes that are not mutually exclusive and often co-exist. These observations might also underly DTP cell phenotypic heterogeneity, such as slow or absent cellular proliferation, different metabolic alterations

and/or variable interactions with the tumour microenvironment (Fig. 3). Developing strategies that directly target these DTP-associated features with the goal of either suppressing the effects of DTP cells or attempting to cure the cancer in general might yield more-effective therapeutics that ameliorate cancer drug resistance.

## **[H2] Eradicating DTP cells by targeting collateral vulnerabilities**

The various traits acquired by DTP cells probably come at the expense of certain other properties, resulting in a trade-off known as ‘collateral sensitivity’, at least in certain circumstances<sup>141</sup>. This effect can create a therapeutic opportunity where an emergent vulnerability can be exploited in a treatment-specific context (Fig. 3, (1)). Various strategies can be used to target the collateral vulnerabilities of DTP cells. Iron-dependent metabolism is a hallmark characteristic of DTP cells<sup>142</sup>, making them collaterally sensitive to ferroptosis, a nonapoptotic form of cell death arising from the iron-mediated production of oxygen radicals<sup>45, 103</sup>. Data from preclinical experiments demonstrate the ability of ferroptosis induction to kill cancer cells<sup>142, 143</sup>, although such a strategy has not been tested clinically. Erastin is one of the most studied ferroptosis inducers, inhibiting the cysteine/glutamate transporter system Xc, composed of SLC7A11 and SLC3A2 amino acid transporters<sup>144</sup>. Inhibition of cysteine/glutamate transporter system Xc results in reduced production of glutathione which is a cofactor of GPX activation, leading to a reduction in the cell’s antioxidant capacity and hence increased L-ROS, and ultimately cellular ferroptosis<sup>145</sup>. This agent has been shown to induce ferroptosis across a variety of cancer cell lines<sup>146</sup>. However, erastin and related compounds have limited solubility, thus limiting interest in their further clinical development. Another inhibitor of cysteine/glutamate transport, sulfasalazine, has been shown to induce ferroptosis via upregulation of MESH1, a cytosolic NADPH phosphatase. Inhibition of MESH1 leads to NADPH depletion and thus sensitizes cells that are reliant on iron-dependent metabolism to ferroptosis<sup>147</sup>. Sulfasalazine is already approved by the FDA for patients with rheumatoid arthritis<sup>148</sup>, making this agent a candidate for further evaluation in DTP-related preclinical models and clinical trials given its established safety profile. RSL3, another ferroptosis inducer, inhibits the glutathione peroxidase GPX4, resulting in specific eradication of DTP cells<sup>103</sup>. However, similar to erastin, this compound is still in preclinical development. Further studies are needed to determine the potential of these agents for therapeutic modulation of DTP cells in clinical settings.

Targeting acquired epigenetic alterations provides another potential therapeutic DTP cell modulation strategy. For example, targeting the H3K4 demethylase KDM5 has been

shown to eradicate persister cells in cell lines derived from melanoma, NSCLC and breast cancers<sup>149</sup>. High-throughput screening identified the lead compound CPI-455, which increases H3K4me3 levels and decreases the number of DTP cells in these preclinical models. Modulating KDM5 activity could potentially decrease the transcriptomic heterogeneity of cancer cells, which might impair the emergence of DTP cells and improve responses to endocrine therapy in breast cancer cell lines<sup>150</sup>. Conversely histone deacetylase (HDAC) inhibition can potentially de-repress long interspersed repeat 1 elements (comprising approximately 20% of the genome), leading to the eradication of DTP cells in PDX models of NSCLC<sup>78</sup>. HDAC inhibitors, such as vorinostat and belinostat, are FDA-approved therapies for patients with T cell lymphoma ([https://www.accessdata.fda.gov/drugsatfda\\_docs/nda/2006/021991s000\\_zolinzatoc.cfm](https://www.accessdata.fda.gov/drugsatfda_docs/nda/2006/021991s000_zolinzatoc.cfm))<sup>151</sup>, and have, or are being, investigated in combination with other agents as first-line therapies various clinical trials involving patients with solid tumours (*ClinicalTrials.gov* ID: *NCT01413750*<sup>152</sup> and *ClinicalTrials.gov* ID: *NCT04674683*). However, the rationale for these trials was based on increasing either the immunogenicity of cancer cells or stimulating the anticancer activity of immune cells: none of these strategies were designed to specifically target DTP cells.

Collateral vulnerability-based strategies might be worth exploring in other cancer types. DTP cells derived from *EGFR*-mutant NSCLC cell lines have increased nuclear YAP localization and dynamic alterations in chromatin accessibility<sup>153</sup>. Data from drug screens indicate that these residual tumour cells are sensitive to MEK, AURKB, BRD4 and TEAD inhibitors<sup>153</sup>. Elsewhere, inhibition of Notch signalling, potentially combined with inhibition of histone demethylases KDM6A/B has been shown to eliminate glioblastoma cells that have entered a stem-like DTP state following in vitro exposure to tyrosine-kinase inhibitors<sup>37</sup>. The stem cell-like identity<sup>154</sup> adopted by a subset of melanoma cells, relying on a glial cell line-derived neurotrophic factor-dependent signalling cascade, could be effectively targeted by FAK inhibitors in preclinical models, which delayed the emergence of acquired resistance<sup>28, 155</sup>. These agents, potentially in combination with SOC first-line therapies or in a sequential regimen designed to target collateral vulnerabilities, could be tested in future clinical trials (Fig. 3, (1)).

## **[H2] Avoiding DTP cell re-activation.**

Counterintuitively, maintaining DTPs in a slowly-proliferating or potentially quiescent state for an extended duration could delay resistance and increase the period of time in which

targeted eradication of DTP cells, either by exploiting specific vulnerabilities or activating antitumour immunity, is feasible. For example, DTP cells derived from preclinical models of *EGFR*-mutant NSCLC can enter a senescence-like state following sustained exposure to osimertinib combined with inhibition of downstream signalling with the MEK inhibitor trametinib. Exposure to this combination results in transcriptional repression of the pro-apoptotic protein Bmf, increased expression of senescence-associated genes, increased secretion of several senescence-associated secretory phenotype (SASP) factors, and induction of p27<sup>kip</sup> and punctate H3K9me3-positive nuclear foci<sup>156</sup>. However, a drug-induced SASP-associated permissive microenvironment can have both positive and negative effects on residual cancer cells, including the promotion of a DTP-associated cellular phenotype and DTP cell survival, while also promoting antitumour immunity in preclinical models of lung and pancreatic cancers<sup>157, 158</sup>. This microenvironment might also be age-dependent and organ-specific. For example, WNT5A signalling maintains residual melanoma cells in a dormant state until age-related secreted frizzled related protein 1 (sFRP1) inhibits the WNT5A signalling axis, leading to the re-activation of residual cancer cells in the lung tissues of aged (>52-week-old)-mouse models<sup>159</sup>. Similarly, activation of the GAS6–AXL signalling axis can inhibit the reactivation of residual DTP cells mediated by sFRP1, as seen in preclinical models of NSCLC<sup>91</sup>. CDC7 inhibition can also induce senescence-like growth arrest. For example, pharmacological inhibition of CDC7 in hepatocellular carcinoma cell lines suppresses homologous recombination repair, delays DNA damage recovery and maintains residual cells in a dormant state. This approach also sensitizes the cells to chemotherapy or mTOR inhibition<sup>160, 161</sup>. These observations suggest that investigations should focus on therapeutic interventions that simultaneously achieve cytoreduction and avoid DTP cell reactivation. A phase I trial testing the CDC7 inhibitor, LY3143921 as monotherapy in patients with advanced-stage *TP53*-mutant solid tumours, including squamous NSCLC, high-grade serous ovarian cancer, TNBC and pancreatic cancer, is ongoing (*ClinicalTrials.gov* ID: *NCT03096054*) (Fig. 3, (2)).

## **[H2] Adaptive therapy to contain DTP cells**

Combining treatments that simultaneously target both treatment-sensitive cancer cells and DTP cells has the potential to substantially improve survival outcomes. However, combination regimens often come with a high risk of toxicities. To address this challenge, adaptive therapy is emerging as a viable option in patients with advanced-stage cancers<sup>162, 163</sup>. Adaptive therapy initially exploits mechanisms of acquired resistance that often come with

certain trade-offs, such as reduced proliferation, allowing drug-sensitive populations to outcompete resistant populations in a drug-free environment<sup>164</sup>. However, administering cytotoxic drugs at the maximum tolerated dose until the emergence of radiographically detectable disease progression strongly selects for resistant clones, leading to rapid proliferation of resistant populations. Ideally, appropriate intermittent treatment can enable residual DTP cells to re-enter into a cell state that is proliferative and sensitive to the same treatment<sup>165</sup>. This approach could prolong the effectiveness of a drug by delaying the emergence of acquired resistance (Fig. 3, (3)). Data from preclinical studies demonstrate that intermittent treatment response can provide improved tumour control<sup>166</sup>. Two clinical studies conducted head-to-head comparisons between continuous and intermittent treatment regimens in advanced melanoma. Algazi et al. conducted a phase II clinical trial (*ClinicalTrials.gov ID: NCT02196181*) that randomized patients benefiting from targeted therapy after an 8-week lead-in period, during which the patients received continuous treatment of dabrafenib and trametinib. Subsequently, patients randomized to intermittent dosing had an off-treatment interval of three weeks until clinical imaging response assessments at 8 weeks<sup>167</sup>. The Spanish melanoma group also performed a similar phase II randomized study (*ClinicalTrials.gov ID: NCT02583516*) to evaluate the anti-tumour efficacy (primary endpoint: PFS) of continuous versus intermittent administration of vemurafenib and cobimetinib in combination, with an exploratory cfDNA from longitudinal plasma samples as a secondary endpoint<sup>168</sup>. Adaptive therapy is thought to alternate between therapy-driven pressure against drug-sensitive cells during periods of drug treatment and regrowth of DTP cells upon the removal of therapy-mediated selection pressure. In this manner, DTP cells could potentially re-enter into a sensitive cell state that may be a dominant mechanism underlying the efficacy of the intermittent schedule. Hussain et al. compared one specific intermittent therapy regimen, a hormonal adaptive therapy with abiraterone, and demonstrated similar clinical outcomes in a large phase III study (*ClinicalTrials.gov ID: NCT00002651*) in castration-resistant prostate cancer<sup>169</sup>. Another study (*ClinicalTrials.gov ID: NCT02415621*) enrolled an intermittent therapy approach that aims to avoid the emergence of treatment resistant cells using mathematical modelling informed by patient-specific tumour dynamics, and this seems to be effective in terms of delaying disease progression, although the cohort is small and there seems to be no control arm<sup>170</sup>. Therefore, future personalized intermittent therapy may be an effective treatment strategy by taking into account the resistance dynamics of individual tumours and the dynamics of DTP cells.

## **[H2] Developing clinical trials targeting DTPs**

To develop well-designed clinical trials evaluating interventions targeting DTP cells, investigators will need to reconsider the currently used clinical end points. Radiographic evaluations of end points such as objective response rate or progression-free survival might not accurately reflect the residual disease state at best response. Combining treatments to target both proliferating cancer cells and DTP cells will require alternative radiological imaging methods and/or the analysis of longitudinally obtained liquid biopsy samples that enable the detection and monitoring of residual disease. This need for DTP-specific end points and novel methods of evaluating them is a crucial challenge for researchers and medical oncologists with an interest in DTP cells. If successful, the availability of accurate methods of DTP cell detection and monitoring would dramatically improve our understanding of therapeutic tolerance and resistance. The European PERSIST-SEQ research consortium is expected to drive research in this direction with the goal of developing novel targeted prevention and mitigation methods and thus overcome cancer therapeutic resistance (<https://persist-seq.org/>). Among other avenues of research, clinical trials testing neoadjuvant therapies have the valuable advantage of providing surgical specimens, thus enabling investigations of clinically relevant biological mechanisms of residual disease and the identification of possible biomarkers. Only by studying both *in vitro* and *in vivo* cancer models and using such data to inform the design of clinical trials testing DTP cell-related interventions can we translate knowledge of biological mechanisms into clinical advances. Data from *in vivo* and/or *in vitro* models could provide a solid biological rationale for combining treatments in concurrent, sequential or alternating regimens that maximize efficacy and minimize the incidence of adverse effects.

## **[H1] Conclusions**

Complete and durable responses to SOC therapies in patients with advanced-stage solid tumours remain rare. DTP cells, whether microbial or human, serve as a reservoir for the evolution of cells harbouring stable mechanisms of drug resistance, making them attractive targets for novel curative therapies. However, the current gap in the availability of experimental versus clinical data hinders the translation of DTP cell targeting interventions from bench to bedside. Future work in this domain should prioritize collaborations between researchers, medical oncologists and those from other clinical specialties in the collection and interpretation of longitudinal samples that will enable improvements in our understanding of the biology of persistence in clinical settings. Trials testing DTP cell-directed clinical



interventions, in combination with cutting-edge analysis techniques such as single-cell sequencing, tissue and liquid biopsy profiling and mass spectrometry-based assessments, are expected to enable extensive characterization of DTP cells and their associated tumour microenvironments in clinical samples. A deeper understanding of the biology of cancer DTP cells will enable the development of novel therapies that counter the effects of these deadly survivors and, consequently, induce durable responses.

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## **Author contributions**

All authors made a substantial contribution to all aspects of the preparation of this manuscript.

## **Competing interests**

C.R. has acted as a consultant for Astra Zeneca, BMS, MSD, Pfizer, Pierre Fabre, Roche and Sanofi and is a co-founder of Ribonexus. Y.P., L.L., H.P., L.L., D.H., F.V. and S.S. declare no competing interests.

- Drug-tolerant persister (DTP) cells are phenotypically heterogeneous, with diverse characteristics such as slow-proliferating or proliferating phenotypes, and a range of metabolic adaptations, cell identity switches and immune-evasion mechanisms. The specific traits expressed depend on the type of cancer and the treatment received.
- DTP cells arise stochastically. All cancer cells have the potential to enter a drug-tolerant state depending on their initial adaptive response to treatment.
- The DTP cell phenotype is transient and does not reflect the presence of resistance mutations. These cells can either produce drug-sensitive progeny or evolve toward irreversible, acquired resistance mediated by acquired genomic alterations.
- DTP cells emerge in tumours that initially respond to systemic anticancer therapies and might not be involved in disease recurrence following local therapies such as surgery. Incomplete responses to systemic therapies might reflect the coexistence of several distinct populations of resistant cancer cells, including dormant tumour cells, CSCs, clones harbouring primary resistance and DTPs.
- No universally accepted biomarkers enabling the identification of DTP cells currently exist. The roles of DTP cells should be investigated in the context of specific clinical scenarios to facilitate their optimal application in precision oncology.

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Figure 1

**Figure 1. Persistence of drug-tolerant persister (DTP) cells.** Neoadjuvant therapy followed by surgery and systemic adjuvant therapy can dramatically reduce tumour burden to levels not detectable using standard clinical methods, potentially with undetectable minimal residual disease (MRD), which can be comprised of a phenotypically heterogeneous population of drug-tolerant persister (DTP) cells. Cancer cells can stochastically switch between sensitive and primed 'pre-DTP' states prior to treatment, and a percentage of these pre-DTP cells will enter a bona fide DTP cell state upon exposure to treatment. DTPs are phenotypically heterogeneous depending on their interactions with a specific treatment. Upon treatment discontinuation, DTP cells can produce treatment-sensitive progeny that retain certain properties of their parent cells, or might also acquire resistance mutations or other stable mechanisms of resistance, resulting in repopulation of the tumour with clones that are resistant to the previously received treatment.

Figure 2

**Figure 2. Detection of drug-tolerant persister cells in clinical settings. a.** Iron-dependent metabolism is a shared feature of drug-tolerant persister (DTP) cells derived from various solid tumours. Iron uptake can be mediated by various membrane transporters or receptors, including transferrin receptors (TFR1), SLC11A2 and CD44. These pathways enable the clinical detection of DTPs using alternative radiotracers, such as  $^{68}\text{Ga}$ -citrate or  $^{68}\text{Ga}$ -labelled fibroblast-activation protein inhibitor (FAPI). PET-CT with a  $^{68}\text{Ga}$ -FAPI-46 radiotracer can enable the detection of residual tumour cells that are not dependent on glycolysis in a patient with nasopharyngeal cancer after concurrent chemoradiotherapy. Coronal and axial images demonstrate focal  $^{68}\text{Ga}$ -FAPI-46 uptake in the absence of  $^{18}\text{F}$ -FDG uptake in a suspected residual bone metastatic lesion located in the ninth right lateral rib<sup>110</sup>. Adapted from *J Nucl. Med.* 2022; 63, 1155-1161110. **b.** Novel liquid biopsy technologies can be applied to the detection of circulating DTP (cDTP) cells and DTP-derived circulating cell-free tumour DNA (ctDNA). Selection based on putative DTP cell-specific biomarkers, such as CD36, can be combined with immunoaffinity-based enrichment for the isolation of cDTPs from blood samples. DTP cell-associated epigenetic modifications can also be used for the identification of DTP-derived specific ctDNAs. Owing to the minimal invasiveness of sampling, such analyses can be used to longitudinally monitor tumour evolution during the course of treatment, although clinical assays designed specifically for the purpose of DTP cell detection are currently unavailable.

## Figure 3

**Figure 3. Potential drug-tolerant persister cell-targeting therapeutic strategies.** (1) Targeting the collateral vulnerabilities of DTP cells by administering combinations of systemic agents to directly eradicate residual tumour cells. (2) Sequential treatment with agents designed to avoid or delay the reactivation of dormant DTP cells, thus maintaining a minimal tumour burden. (3) Adaptive therapy to avoid or delay the emergence of DTPs while maintaining a moderate tumour burden. Delivering effective adaptive therapy in a clinical setting remains challenging. Nonetheless, using a multidisciplinary approach to determine the optimal treatment dose and interval lengths in the context of the dynamic evolution of DTP cells could further advance the development of this strategy.

### **BOX 1. Bacterial persisters and cancer DTP cells display biphasic kinetics after initial exposure to treatment**

Persister cells can be detected using simple cell death assays in which a sensitive population of **a.** bacterial cells or **b.** cancer cells is exposed to antibiotics or anticancer drugs, respectively. Biphasic cell death dynamics, featuring the initial death of the majority of the population combined with delayed reductions in the resistant population are observed in both bacterial persister<sup>177</sup> and cancer cell populations<sup>69</sup>. (1) Lethal doses of treatment administered at time zero to an exponentially growing population of sensitive, genetically identical cells. (2) The first phase of log-linear killing slope (depicted by the bold black dashed line) is characterized by exponential kinetics, in which the susceptible subpopulation quickly dies. (3) In parallel, the slower death kinetics seen in the second phase (depicted by the dashed red line) reveal the existence of a persistent subpopulation that emerges after the susceptible subpopulation is eliminated. In bacteria, estimates of the slope of the second phase likely reflect the percentage of tolerant cells in the pretreatment population, whereas, in populations of cancer cells, drug-tolerant persister (DTP) cells can emerge de novo<sup>69</sup>. The grey dashed line indicates the predicted growth kinetics of a heterogeneous, highly resistant population of bacteria or cancer cells. CFU, colony forming unit; FC, fold change. Adapted from *Nat Genetics*, 2022; 54, 976-984.

## **BOX 2. Drug resistance and drug-tolerant persister cells in cancer**

The phenomenon of reversible drug tolerance has been observed in clinical reports describing patients with cancer receiving various anticancer therapies<sup>171</sup>, wherein re-commencing treatment with the same drug after a ‘drug holiday’ due to either drug resistance or tolerability is clinically beneficial<sup>172, 173</sup>. The monitoring and targeting of drug-tolerant persister (DTP) cells in clinical practice presents potential advantages over traditional therapy regimens, including expanded therapeutic options, and may eventually lead to improvements in patient’s quality of life. DTP cells are a subset of cancer cells that are able to survive exposure to anticancer treatments and remain undetected until they result in clinical progression. A wide range of mechanisms explaining this persistence phenomenon have been identified, although the high phenotypic heterogeneity of DTP cells necessitates careful contextual definition. Nevertheless, DTP states share certain common characteristics that are specifically reflected in their interactions with particular treatment regimens, possess a stochastic origin, and are in a dynamic cellular state with the ability to develop permanent acquired resistance.