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Perspective

Drug-tolerant persister cancer cells

Pengliang Wang [4,†], Bin Ke [1,2,3,†], Gang Ma [1,2,3,*]

[1] Department of Gastric Surgery, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Tianjin, China
[2] Tianjin Key Laboratory of Digestive Cancer, Tianjin, China
[3] Tianjin's Clinical Research Center for Cancer, Tianjin, China
[4] Department of Gastrointestinal Surgery, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, China

Drug resistance continues to be a major bottleneck of curing patients with cancer. Two primary intracellular factors contribute to emergence of drug resistance in treatment-naïve cancer cells, which are a population of malignant cells not receiving any therapy before. One is that genetic mutations occur in key cancer-addicted genes, such as T790M in epidermal growth factor receptor (EGFR), are a critical mechanism underlying some therapeutic resistance, thus boosting the development and usage of targeted inhibitors to benefit patients with cancer. In addition, non-genetic factors, including aberrant epigenetic/metabolic machinery and/or reprogrammed transcription profile, probably play more important roles in drug resistance in cancer cells.[1] Upon receiving treatment with osimertinib, a subset of EGFR-mutated lung cancer cells acquired resistance to osimertinib through mammalian SWI/SNF complex-mediated alterations in chromatin accessibility to sustain low cellular ROS level and hyperproliferation, but no new mutations occurred in EGFR.[2]

Recently, researchers observed that a small group of cancer cells survived the treatment with lethal dose of chemotherapeutic or targeted drugs, and re-entered cycling phase and prospered after drug withdrawal.[3] Furthermore, these flourished cell populations remained sensitive to originally administrated drugs and no additional genetic mutations in critical resistance-related genes occurred, whereby these cells are named after drug-tolerant persister cancer (DTPC) cells.[3] Notably, a case report showed that DTPC cells were responsible for relapse in one patient with lung adenocarcinoma (LUAD), who had received gefitinib, thus highlighting significance of drug tolerance in clinical treatment and entailing a deeper understanding of DTPC cells and identification of therapeutic vulnerabilities.[4] Whereas as of yet there is no consensus in oncology community about the definition of drug tolerance in cancer cells, we want to emphasize that plasticity is its core property (Fig. 1). In contrast, drug resistance is usually a phenotype that cancer cells become stably non-responsive to drugs, at least compared with DTPC cells. Additionally, since drug tolerance is highly plastic, it is plausible to observe that this population reverses to originally "drug-sensitive" state or drug-resistant populations develop from within. Therefore, this *Perspective* will describe the characteristics of DTPC cells and underscore their plastic trait.

1. The 1st question: What are DTPC cells?

Cancer plasticity is known as the ability of malignant cells to acquire a continuum of phenotypic states.[5] It is well established that cancer plasticity contributes a lot to drug resistance. A notorious example is that neuroendocrine transdifferentiation emerged in prostate and lung cancer to make cancerous cells become refractory to originally effective drug regimens.[5] The transition among these diverse phenotypes is primarily determined by distinct epigenetic and transcriptional programs, which, unlike genetic changes, are capable of guaranteeing quick adaption to intra- and extra-cellular stresses.[5] According to current findings in this field, drug-tolerance state, in our opinion, is a manifestation of plasticity when cancer cells are challenged by drug insults. Whole exon sequencing analyses did not find additional mutations related to drug resistance in multiple DTPC models.[6,7] In the in vitro models, DTP state usually emerged from drug treatment with no longer than 14 days and vanished after around 2-week drug holiday,[6,7] this reversible phenotype which indirectly demonstrates that drug tolerance of cancer cells is a quick response to therapeutic insults. Additionally, epigenetic determinants dictate the reversible drug-tolerance feature of DTPC cells. Histone demethylase KDM5A expression was markedly increased and supported drug-tolerance state in malignant cells of several cancer types.[3] As a demethylase of histone H3K4me2/3, increased KDM5A reduced levels of these two modified H3K4 and thereby led to global changes in chromatin structure.[3] H3K4me3 is an extensively studied histone marker of activated transcription, and a recent study reported that most tolerance-related genes in triple-negative breast cancer were in a bivalent chromatin modification state, which was characteristic of simultaneously possessing H3K4me3 and transcription-repression histone modification (H3K27me3).[8] This bivalent chromatin configuration is usually found at the promoters of differentiation genes during mammalian development, which favors the dynamic and timely regulation of gene expressions.[9] Intriguingly, loss of H3K27me3 was the early event happened at the beginning of chemotherapy, which then activated epithelial-mesenchymal transition (EMT) program and NF-κB signal pathway in DTPC cells; thus, preservation of H3K27me3 remarkably prevented formation of DTPC cells.[8] The distinct roles between H3K4me3 and H3K27me3 is probably

* Corresponding author.
E-mail address: horsegang@tmu.edu.cn (G. Ma).
† These authors contributed equally to this work.

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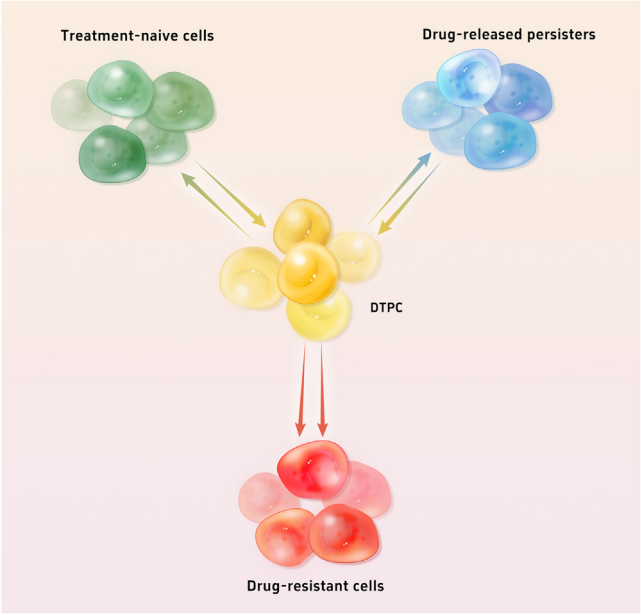

Fig. 1. The reversible/irreversible transitions among treatment-naïve cells, DRP cells, DTPC cells, and drug-resistant cells. A small portion of treatment-naïve cells could evolve into DTPC cells when they are subjected to chemotherapeutic drugs (e.g., cisplatin) and small molecule inhibitors (e.g., TKIs), while DTPC cells have the potential to revert to treatment-naïve cells or DRP cells depending on different experimental settings. DTPC cells in different models display various gene expression profiles, such as diapause-like, EMT-like and fetal stem cell-like state. Thus, DTPC cells, as a group, are highly heterogenous. DRP cells, although they manifest the similar phenotypes as treatment-naïve cells, are inherently distinct from the latter, as evidenced by the fact that DRP cells become more inclined to turn into DTPC cells. On receiving prolonged drug treatment, drug-resistant cells equipped with diverse resistant mechanisms emerge from DTPC cells irreversibly. The differences in color shades and cellular shapes within the same groups of populations represent cellular heterogeneity. DRP cells, drug-released persister cells; DTPC cells, drug-tolerant persister cancer cells; EMT, epithelial–mesenchymal transition; TKI, tyrosine kinase inhibitor.

due to variations in cancer contexts. Collectively, these findings strongly suggest that epigenetic machinery is the core regulator in the formation and maintenance of drug-tolerance state.

Metabolism can be timely rewired to supply fundamental biological molecules (DNA/RNA, proteins, lipids and saccharides) and sustain proper intracellular ROS level in response to constantly changing extracellular environments.[10] Meanwhile, metabolism influences chromatin states by offering key intermediates such as methyl/ethyl groups.[10] Thus, it is not surprising that metabolism is reprogrammed in DTPC cells compared to treatment-naïve cells. Acetyl choline (Ach) was enriched in DTPC cells derived from non-small cell lung cancer (NSCLC) and essential for maintaining drug-tolerance state, which was due to YAP-regulated increased expression of choline acetyltransferase that is the rate-limiting enzyme to catalyze Ach biogenesis.[11] Another study of LUAD cells showed that glycolysis was switched to oxidative phosphorylation (OXPHOS) via mitophagy controlled by PTEN-induced kinase 1 (PINK1) in DTPC cells, and knockdown of PINK1 expression delayed the emergence of DTPC cells.[12] A somewhat conflicting result is reported that colorectal DTPC cells displayed enhanced glycolysis activity rather than OXPHOS.[13] The seemingly contradictory results may be that the colorectal DTPC cells assumed diapause-like state,[13] which was not observed in the DTPC cell population arose from LUAD. Diapause is a biological process that embryos enter a reversible dormancy when their environments become adverse, and high glycolysis activity is a prominent metabolic feature of this program.[14] Notably, the increased level of lactate, as the result of enhanced glycolysis, altered lactylation modification of histone lysine residues, such as H4K12, and promoted the expressions of several members of ABC transporter family to pump drugs outside.[13] Anti-cancer drugs, especially for traditional chemotherapy drugs, usually increase intracellular ROS level to exert their tumoricidal effect. DTPC cells boosted NRF2 signal pathway and glutathione metabolism to tackle potentially harmful oxidative stress.[15] In addition, Niemann-Pick C1 like 1 (NPC1L1) expression was highly elevated in DTPC cells, and then the protein facilitated the uptake of more vimentin E from outside, which removed lipid peroxyl radicals to decrease oxidative stress.[16] These results together corroborate that DTPC cells employ interrelated epigenetic and metabolic machinery to survive drug insults.

A third vital property in common in most DTPC cells is evasion from apoptosis induced by chemotherapeutic drugs and/or targeted inhibitors.[17] Treatment with pro-apoptosis BH3 mimics, such as ABT-737 and S63845, triggered cytotoxic effects in lung cancer cells; however, a small portion of cells did not succumb to apoptosis but acquired DTP features and survived instead.[18] In these drug-tolerant cells, BH3 mimics triggered release of inadequate amount of cytochrome c from mitochondria, which thereby failed to assemble apoptosome; on the contrary, the released cytochrome c was sensed by heme-regulated inhibitor (HRI) kinase and then activated integrated stress response (ISR) signal pathway to drive drug-tolerance phenotype.[18] Additionally, increased expression of several anti-apoptosis genes was identified in DTPC cells. On receiving tyrosine kinase inhibitors (TKI), NSCLC-originated DTPC cells displayed markedly increased MCL1 protein level. Mechanistically, mTORC2 phosphorylated MCL1 at residue Ser64 to promote bind of MCL1 to BIM in these cells, thus facilitating the escape of MCL1 from ubiquitin-mediated degradation.[19] Cellular inhibitor of apoptosis protein 2 (cIAP2) expression was increased at transcription level, promoted tolerance to MEK inhibitors, and reduced apoptosis in melanoma cells.[20] Accordingly, DTPC cells probably employ distinct mechanisms to resist apoptosis, which provides a therapeutic opportunity for eradicating them.

2. The 2nd question: Where are DTPC cells from?

Whereas DTPC cells share the relatively similar phenotypes and molecular traits as described above, these DTPC cells under investigation are barely homogenous populations (Fig. 1). This heterogeneity does not originate from genetic variations but from distinct transcription profiles. To date, it has been becoming increasingly evident that treatment-naïve cancer cells usually hijack normal development programs to acquire the capacity of drug tolerance. RNA sequencing unraveled that colorectal cancer (CRC) cells manifested a diapause-like gene signature, which was characteristic of downregulated MYC/mTORC1/cell cycle signal pathway, when these cells were exposed to 5-fluorouracil (5-FU), oxaliplatin, or 5-FU and oxaliplatin (FOLFOX).[6] Hostile environments are critical in triggering diapause in mammalian embryos under development. Similarly, CRC HCT116 cells displayed typical diapause downregulation gene signature, including attenuated cell cycle signal pathway, in culture medium deprived of serum or glucose.[13] Among these downregulated genes, structural maintenance of chromosomes 4 (SMC4) was found to be enriched in the fast-cycling MC38 cell population and play key roles in the switch to diapause-like state, since knockdown of SMC4 expression inhibited the proliferation and exacerbated tolerance to irinotecan.[13] Nevertheless, adverse stem cell niche does not initiate diapause-like program. Removal of epithelial growth factor (EGF) or addition of transforming growth factor-beta (TGF-β) did not induce diapause; instead, this niche heightened Mex3a expression in a subset of Lgr5$^+$ CRC cells, and slowed down their proliferative rate.[21] More importantly, Lgr5$^+$/Mex3a$^+$ cell population was a group of DTPC cells, which was tolerant to 5-FU, 7-Ethyl-10-hydroxycamptothecin (SN38) or oxaliplatin, and its expression profile was similar to that of fetal stem cells.[21] Lapatinib, as a clinically used small inhibitor targeting epithelial growth factor receptor 2 (HER2), induced drug-tolerance phenotype in several HER2$^+$ breast

cancer cell lines (e.g., BT-474), however it failed to recapitulate the same phenotypic transition in other subtypes of breast cancer cells.[22] Moreover, RNA-sequencing showed that lapatinib-induced DTPC cells were clustered into two distinct groups, one of which possessed EMT-like gene signature while another had transcription profile of positive estrogen response.[22] Accordingly, formation of DTPC cell populations is subjected to different cellular programs, this heterogeneity which are probably determined by diverse cancer contexts, drug types and experimental settings.

The next issue needed to be addressed is whether this drug-tolerance switch is stochastic or pre-determined. Under the former conditions, most treatment-naïve cells, if not all, could become DTPC cells, while in the latter scenario only a small population of cells acquire drug-tolerant capacity under therapeutic pressures. Cellular barcoding experiments demonstrated that clonal complexity did not significantly change between treatment-naïve and DTPC cells, thus strongly suggesting that DTPC cells did not evolve from a subset of cells with selective growth advantages.[6] Using the same technology, researchers confirmed that $HER2^+$ breast cancer cells had equal potency to become DTPC cells in the presence of lapatinib.[22] But a piece of inconsistent finding was that $Mex3a^+$ CRC DTPC cells originated from a small population of $Lgr5^+$ CRC cells, as evidenced by the fact that Mex3a expression was markedly increased in only 10 % $Lgr5^+$ cells resided in normal colonic mucosa and these $Lgr5^+/Mex3a^+$ cells displayed drug-tolerant feature upon exposed to chemotherapeutic drugs.[21] The researchers claimed that this result did not rule out the possibility of other $Mex3a^-$ cells to turn to be DTPC cells.[21] In our opinion, therapeutic strategies and intervening targets are likely to introduce variations into the observed responses in DTPC cells. One study showed that drug types determined the chance to become DTPC cells, as cytotoxic therapeutic drugs cultivated a broader pan-drug tolerance than those target-specific inhibitors.[23] However, these findings underscore the importance of cellular plasticity in the formation of DTPC cells.

3. The 3rd question: Where are DTPC cells going?

Current findings demonstrate that DTPC cells have two fates. One is that a subset of DTPC cells re-enter cell cycling stage without acquired drug resistance after drug holiday. Single-cell sequencing demonstrated that there were non-cycling and cycling population in the late DTPC cells, and these two groups of cells had distinct transcription programs.[15] The transcription landscape of the cycling population was reminiscent of that of the drug-naïve population.[15] These cycling cells eventually regenerated a new population, also called as drug-released persisters (DRPs) by researchers, upon drug withdrawal, and DRPs harbored significantly increased proportion of the subpopulation that were predisposed to DTPC cells, implying that DRPs possessed inheritable machineries to maintain the capacity of switching to drug-tolerance state (Fig. 1).[23] Another destiny of DTPC cells is transformation into drug-resistant cells (Fig. 1). Whereas there is no consensus about its definition in academic circle, drug tolerance in cancer is inherently different from drug resistance. Drug tolerance is more like a first aid strategy adopted by cancer cells in the presence of drug insults. On the other hand, prolonged drug treatment would yield irreversibly resistant populations,[6] which eventually become dominant clones in tumor mass. More importantly, drug-resistance cell populations could emerge from DTPC cells and these cells had various drug-refractory mechanisms,[24] further suggesting that drug tolerance itself did not hinder the evolvement of drug resistance. For example, *BCL2* amplification was usually detected in mantel cell lymphoma, however, 18q21 amplicons, which harbor *BCL2*, was lost in the resistant cell population derived from DTPC cells induced by Venetoclax (ABT-199), a small molecule inhibitor of BCL2.[25] This finding indicated that DTPC cells are equipped with intrinsic machineries to drive occurrence of genetic aberrations in the conversion from drug tolerance to drug resistance. One key player is APOBECs. This group of deaminases convert cytidine into uridine to lead to C→T and C→G, these genetic aberrations which are known as APOBEC mutational signature. As one member of APOBEC family, APOBEC3A (3A3) expression was markedly increased to exacerbate APOBEC mutations and frequency of double strand breaks (DSB) in osimertinib-induced NSCLC DTPC cells, thus activation of 3A3 is at least partially responsible for genomic instability in TKI-resistant cells.[7] Analysis of clinical samples also validated that longer duration of osimertinib treatment heightened APOBEC mutation frequency.[7] Another critical regulator is receptor tyrosine kinase AXL, whose expression was aberrantly increased in multiple malignancies. It has been described that AXL contributed greatly to intrinsic resistance to osimertinib in EGFR-mutated lung cancer cells.[26] Meanwhile, AXL promoted survival of DTPC cells and formation of drug-resistant clones.[27] This effect of favoring drug resistance relied on two major mechanisms, one of which was accelerated error-prone DNA replication to facilitate occurrence of typical drug-resistant mutations, such as $EGFR^{T790M}$, and the other of which was MYC-dictated reprogrammed cellular metabolism.[27] These results validate that drug tolerance is highly dynamic and might be a stage prior to drug resistance.

4. Candidate strategies to tackle DTPC cells

The exact status of a population of cancer cells determined the regimens.[28] Drug tolerance is probably a valid therapeutic window to prevent emergence of drug-resistant cancer cells. As reported before, DTPC cells develop tolerance to chemotherapeutic and/or targeted drugs. Fortunately, molecular traits uncovered within recent decade provide promising targets for treating DTPC cells. Due to space limit of this manuscript, we do not list all experimental therapeutic results; instead, we would like to briefly describe a framework by categorizing current major strategies into four types (Table 1). The first strategy is to target aberrant epigenetic machinery, which is the principal feature of DTPC cells. KDM5A is the first identified epigenetic regulator to drive emergence of drug tolerance, thus small inhibitors against this enzyme were developed and decreased survival in DTPC cells.[3,29] The changes in the trimethylated status of H3K4/H3K27 also play critical roles during the transition to DTPC cells, thereby inhibitors of KDM6 could eradicate these tolerant tumor cells.[8,30]

In addition, survival of DTPC cells heavily relied on lipid hydroperoxidase GPX4, and inhibitors, such as RSL3 or FIN56, reduced GPX4 activity to trigger ferroptosis in multiple types of cancers.[31] Whereas DTPC cells are capable of evading apoptosis induced by chemotherapeutic drugs, the key proteins that control this process are probably optimally druggable targets. Increased cIAP2 was reported to promote development of DTPC cells, and treatment of cIAP2 inhibitor suppressed onset of DTPC cells in SOX10-dificient melanoma and EGFR-mutated lung cancer cells.[20,32] Similarly, MCL-1 inhibitor could largely recapitulate the effect of cIAP2 inhibitor by re-igniting apoptosis in tolerant NSCLC cells.[19] Thus, programmed cell death mechanisms, such as ferroptosis and apoptosis, are still a therapeutic opportunity of eliminating DTPC cells.

Distinct transcription profiles also provide several valuable targets in treating DTPC cells, as yet CDK7, which was essential for transcription initiation and DNA repair, has been found to sustain drug tolerance state in mantle cell lymphoma and its inhibitor THZ1 predisposed DTPC cells to death through pharmacoproteomic and pharmacogenomic screens.[25] The inhibitory effect of CDK inhibitor on survival of DTPC cells was also confirmed in another study, showing that THZ1 suppressed the transcription activation to support tolerant cancer cells.[33] Except transcription machinery itself, genes with aberrantly increased expression in DTPC cells can be exploited. CD70 is a promising target, since its expression was remarkably upregulated in the early TKI-induced tolerant lung cancer cells and this protein resided in cell surface.[34] Administration of anti-CD70 antibody drug conjugates (Cusatuzumab), CD70-targeting chimeric antigen receptor (CAR) T cell or CAR NK cells significantly ab-

Table 1
Examples of four therapeutic strategies to tackle DTPC cells.

Cancer type	Initial treatment	Potential target	Intervening strategies	Reference
Targeting epigenetic aberrations				
NSCLC	Gefitinib, Erlotinib	KDM5A	Trichostatin A	3
TNBC	5-FU	KDM6	GSK-J4	8
AML	Anthracycline	KDM6	GSK-J4	30
Targeting programmed cell death pathways				
Melanoma	Lapatinib	GPX4	RSL3	31
Breast Cancer	Vemurafenib		ML210	
Melanoma	BRAF inhibitor	cIAP1/2	Birinapant	20
	MEK inhibitor			
NSCLC	Osimertinib	cIAP2	AZD5582	32
NSCLC	Trametinib	MCL-1	GDC-0941	19
	Erlotinib		AZD8055	
	Crizotinib		S63845	
Targeting transcription machinery				
MCL	Venetoclax	CDK7	THZ1	25
Targeting highly expressed proteins in DTPC cells				
NSCLC	Erlotinib	CD70	Cusatuzumab	34
	Osimertinib		CD70-targeting CAR T cells	
			CD70-targeting CAR NK cells	

Abbreviations: 5-FU, 5-fluorouracil; AML, acute myeloid leukemia; DTPC, drug-tolerant persister cancer; MCL, mantle cell lymphoma; NSCLC, non-small cell lung cancer; TNBC, triple-negative breast cancer.

rogated viability in DTPC cells.[34] We propose that research in this field would identify more potential therapeutic targets.

Drug tolerance is a rapidly developing research field, and the findings are constantly shaping the concept of DTPC cells. Some properties of DTPC cells are being recognized and several important issues are required to be addressed. For example, mTOR signal pathway is a regulatory hub in cellular metabolism, however, the functions of this pathway seem intricate in DTPC cells. In a pancreatic cancer model, deactivated mTOR signal pathway was required to sustain survival in drug-tolerant cells.[35] On the contrary, mTOR signal pathway was activated in lapatinib-induced DTPC cells.[22] Therefore, it is necessary to evaluate the roles of intracellular signal pathways in DTPC cells before applying specific inhibitors in clinical practice. However, further investigations of DTPC cells are paramount to developing novel treatment approaches to prevent tumor relapse.

Declaration of competing interest

We declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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G.M. conceived the ideas and acquired funds. P.W. prepared the figure. All of the authors wrote the original draft and conducted the editing.

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