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Publication Date

2018-08-01

DOI

10.1016/j.coisb.2018.03.003

Peer reviewed

HHS Public Access

Author manuscript

Curr Opin Syst Biol. Author manuscript; available in PMC 2019 August 01.

Published in final edited form as:

Curr Opin Syst Biol. 2018 August ; 10: 1–8. doi:10.1016/j.coisb.2018.03.003.

Drug persistence - from antibiotics to cancer therapies

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Abstract

Drug-insensitive tumor subpopulations remain a significant barrier to effective cancer treatment. Recent works suggest that within isogenic drug-sensitive cancer populations, subsets of cells can enter a 'persister' state allowing them to survive prolonged drug treatment. Such persisters are well-described in antibiotic-treated bacterial populations. In this review, we compare mechanisms of drug persistence in bacteria and cancer. Both bacterial and cancer persisters are associated with slow-growing phenotypes, are metabolically distinct from non-persisters, and depend on the activation of specific regulatory programs. Moreover, evidence suggests that bacterial and cancer persisters are an important reservoir for the emergence of drug-resistant mutants. The emerging parallels between persistence in bacteria and cancer can guide efforts to untangle mechanistic links between growth, metabolism, and cellular regulation, and reveal exploitable therapeutic vulnerabilities.

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Declarations of interest: none.

References of special (*) or outstanding (**) interest:

•(*) Brauner et al. [5]: review that provides a conceptual framework to distinguish resistance, tolerance, and persistence

•(**) Levin-Reisman et al. [57]: provides direct evidence that bacterial tolerance precedes the emergence of resistance *in vitro*

•(*) Van den Bergh et al. [55]: stationary bacterial cultures exposed intermittently to antibiotics rapidly evolve to increase rates of persister formation, suggesting that the rate of persister formation is an evolvable trait.

•(**) Sharma et al. [2]: hallmark paper reporting the emergence of a reversible drug-tolerant subpopulation upon treatment of a EGFR-addicted NSCLC cell line with EGFR inhibitors.

•(*) Hata et al. [10] and Ramirez et al. [58]: provide evidence that drug-resistant lung cancer cell populations can emerge from an initial pool of drug-tolerant cells.

•(*) Hangauer et al. [11]: shows that various persister cell models are vulnerable to inhibition of the lipid hydroperoxidase GPX4, presumably through disruption of glutathione metabolism.

•(*) Vinogradova et al. [46] and Roesch et al. [35]: demonstrate that suppressing the activity of KDM5 or KDM6, respectively, inhibits the emergence of drug-tolerant cells in various cancers, highlighting the importance of chromatin-remodeling in the formation of persistence.

•(**) Shaffer et al. [52]: reports existence of transient pre-resistant cell states characterized by sporadic expression of bypassing resistance markers in drug exposed cancer cell populations, suggesting a 'tolerance by sporadic bypassing' mechanism.

•(*) Su et al. [31]: shows that adaptive resistance of patient-derived melanoma cell lines to BRAF inhibition occurs as a cell-state transition to a mesenchymal-like state. Arresting the transition halts the development of drug resistance.

Introduction

The last decades have brought the arrival of an impressive arsenal of therapies for treating cancer. At the same time, countless drug resistance mechanisms have been discovered by which cancer cells avoid and subvert drug treatment. Tumor subpopulations that do not respond to therapeutics are a significant barrier in the treatment of cancer, and cancer remains a major global killer [1].

Recently, it has become clear that even within otherwise drug-susceptible isogenic cancer populations, a subset of cells can enter a persister state, in which they survive prolonged drug exposure [2] (see table 1 for a list of cancer persister models). While this persister state has only recently started to draw attention in mammalian cells, bacterial persisters were described in literature as early as 70 years ago [3]. The past decade has seen a surge in studies elucidating the mechanisms underlying bacterial antibiotic persistence – as recently summarized in a string of excellent reviews [4–8]. In this review, we compare and contrast persistence in bacteria and cancer cells, and highlight surprising parallels in the underlying persistence mechanisms.

1. Defining persistence – a persistent challenge

Before delving into persistence mechanisms, we must first define what drug persistence is, and how it differs from other mechanisms of drug insensitivity (figure 1A).

Bacterial insensitivity to antibiotics is classified phenomenologically into three broad categories that can be distinguished experimentally (compared to a reference sensitive population; figure 1B), as summarized in [4,5]. The first category, **drug tolerance,** is the ability of cell populations to withstand transient lethal antibiotic concentrations, while remaining genetically susceptible [4,5]. Experimentally, tolerance manifests as a decreased rate of killing during drug exposure compared to a sensitive reference population (figure 1B). The second category, **drug resistance**, is the genetically inherited ability of cells to grow at normally lethal antibiotic concentrations [4,5]. Drug-resistant populations show a characteristic increase in minimal inhibitory concentration (the lowest drug concentration needed to prevent bacterial growth); this increase is absent in drug-tolerant populations. In contrast to these two categories, which are defined at the population level, **drug persistence** describes scenarios in which only a subpopulation of cells within a clonal cell population survives prolonged antibiotic treatment, while remaining genetically susceptible to reapplication of the drug [5,9]. An important feature of bacterial drug persistence is its phenotypic reversibility. After drug treatment is stopped, the remaining persister cells will eventually re-establish a population showing the same heterogeneous response when retreated with the same drug (figure 1B). Experimentally, drug persistence is characterized by a survival curve with two phases -an initial steep decline in cell number followed by a cell number plateau -, which is absent in drug-tolerant populations [5,7] (figure 1B).

Compared with the converging literature view of how to define and distinguish bacterial persisters, terminology is somewhat more diverse in cancer literature. Persistence is sometimes used interchangeably with drug tolerance to describe subpopulations that have an enhanced (and non-genetic) ability to survive drug treatment [2,10,11]. Various other terms

have also been used to describe scenarios in which a phenotypically distinct subpopulation survives prolonged drug treatment, including **quiescence** [12], **dormancy** [13] or **cancer stem cells [14]**. *Throughout this review, we will use the term 'persistence' for cases in which a subpopulation survives drug treatment but regains sensitivity after drug removal, and we reserve the term 'tolerance' for cases in which the whole population is more resilient to drug exposure.*

2. Paths to persistence

How do cells become persisters? We will first briefly discuss mechanisms of bacterial antibiotic persister formation, and then relate these to our current understanding of how cancer drug persisters emerge. In particular, we will focus on the impact of three factors on persistence: cell growth, metabolic activity, and regulatory program.

Arguably the best studied bacterial persistence mechanism are Toxin-Antitoxin (TA) systems [15]. These consist of a stable toxin, which arrests growth by inhibiting vital cellular processes such as transcription or translation thereby inducing the persister state, and a labile antitoxin acting as the antidote [7]. An example is the HipBA module in *E. coli*, which inhibits the glutamyl-tRNA synthetase GltX and thus halts translation [16,17]. Originally identified as a mechanism to prevent plasmid loss, TA systems were shown to induce the stochastic formation of non-growing persisters in exponentially growing cultures [18].

Recent works have identified additional factors that modulate antibiotic persistence. For example, various studies found that the fraction of persisters in different environmental conditions is inversely correlated with the population growth rate, as shown e.g. in [19] and summarized in [5]. Additionally, stresses, such as salt-stress, can increase the rate of persister formation [20]. Particularly interesting types of environmental stress are shifts in nutrient availability: bacteria undergoing nutrient shifts, which are typically accompanied by a transient reduction in growth rate, show dramatically elevated persister fractions [21–24]. The examples above evoke a 'tolerance by slow growth' [5] scheme, in which slow-growing bacteria tend to become more resilient against antibiotic treatment, regardless of how exactly the reduction in growth rate came about.

This increase in antibiotic persistence at slow growth could of course simply be the consequence of a reduction in the activity of the antibiotic targets, i.e. the cellular transcription/translation machinery. However, mounting evidence suggests that antibiotic persistence in fact relies on an active cellular program. Various studies have demonstrated that the (p)ppGpp-mediated bacterial starvation program (also termed "stringent response") modulates the rate of persister formation [24–26]. Importantly, mutant strains lacking the stringent response program are readily killed by antibiotic treatment even in starvation conditions [25], suggesting that absence of growth alone is not sufficient to induce persistence.

Finally, recent studies have shown that persisters can be selectively killed off by modulating their metabolic activity [20,25,27,28]. For example, addition of metabolic stimuli that promote an increase in proton-motive force by the oxidative electron transport chain triggers the uptake of aminoglycoside antibiotics in persister cells, thereby enhancing persister

killing [27]. These observations indicate that persisters retain a distinct -and active - metabolic state, and highlight the importance of metabolism for persistence [6,29].

Collectively, these studies paint a picture of bacterial antibiotic persistence with three main themes. The first theme is the recurrent observation that slow bacterial growth tends to favor persister formation. The second theme is a distinct metabolic state in persisters, which leaves them vulnerable to metabolic perturbations. The third theme is the reliance on a specific regulatory program involving the stringent response, which can be directly targeted to reduce persister formation.

Do these themes have parallels in cancer drug persisters? Recent literature suggests that this is indeed the case. The first hint stems from the observation that slow-growing cancer cells also tend to be more drug-tolerant in a wide range of cell types and model systems [12,30–35]. Such drug-tolerance has been reported during exposure to chemo-[35] and targeted [30] therapy, for adherent [32] and suspension [31] cells *in vitro*, as well as *in vivo* in mouse models [12], suggesting a general phenomenon.

Surprisingly, there is also evidence that metabolism might be an important determinant of drug persistence in cancer cells [11,13,35–38]. Recent works by Hangauer et al. and Viswanathan et al. demonstrated that various persister models are vulnerable to inhibition of the lipid hydroperoxidase GPX4 [11,37]. GPX4 catalyzes the glutathione-dependent reduction of lipid peroxides, which cause oxidative stress, and thereby prevents the induction of ferroptosis, a non-apoptotic form of cell death [39]. This result supports earlier work showing that the enzyme aldehyde dehydrogenase 1 A1 (ALDH1A1)—which is also involved in lipid peroxidation and expressed in many cancer stem cells—is required to maintain drug persistence [36]. Moreover, recent reports indicate that persisters rely more heavily on oxidative phosphorylation (OXPHOS), and are more sensitive to OXPHOS inhibitors [13,35,40]. In particular, these subpopulations were reported to have a diminished ‘glycolytic reserve’, which is the ability to increase glucose uptake for ATP generation if OXPHOS is inhibited, suggesting impaired metabolic plasticity [13]. Whether these changes in metabolic activity are an adaptation to redox stress [41,42], or rather reflect an increased demand for ATP [38], is currently unclear.

Finally, several lines of evidence suggest that drug persistence in cancer cells also relies on a distinct regulatory program [43], and particularly pinpoint two regulatory processes. The first process is epithelial-to-mesenchymal transition (EMT), which causes cells to gradually lose their differentiation status and become more stem-cell like [44]. Expression of EMT/stem-cell markers is a frequent hallmark of persister subpopulations, which can be exploited to isolate persister cells within isogenic populations [30,31,35,36,45]. Second, several lines of evidence point towards chromatin remodeling as a key step in persister formation [2,32,46,47]. For example, inhibition of the histone demethylases KDM5 and KDM6 were found to suppress the emergence of persisters [35,46]. These findings are particularly intriguing given that epigenetic and metabolic changes seem to be closely linked in many cancers. A prime example are mutations in metabolic enzymes, such as isocitrate dehydrogenase 1 and 2, which can modulate the epigenetic state of cells through the accumulation of ‘oncometabolites’ such as 2-hydroxyglutarate, and thereby influence cancer

progression and drug survival [48,49]. The involvement of EMT and chromatin remodeling suggests the requirement for a distinct regulatory program to ensure the formation and/or maintenance of a persister state, analogous to the aforementioned stringent response dependent regulation in bacterial persisters. As we will discuss in more detail in the final section of the review, a major open question is how these processes are linked mechanistically in cancer persisters.

Despite these striking parallels, there are also bacterial persistence mechanisms that do not have a direct analog in cancer. For example, a study found that *E. coli* treated intermittently with Ampicillin for different durations quickly evolved population lag times (the time it takes to transition from stationary to exponential growth phase) to match the duration of the antibiotic exposure, while the maximal growth rate did not change [50]. To our knowledge, there is only one example of such 'tolerance by lag' [5] in the cancer literature [51]. Nevertheless, since cancer therapies often involve drug administration at regular time intervals, it is at least conceivable that similar selective pressures might also affect cancer cell populations.

Conversely, there are also cancer persistence mechanisms with no direct bacterial equivalent. A compelling example was recently presented by Shaffer et al [52]. In an elegant set of experiments in patient-derived melanoma cell lines, the authors demonstrated the existence of transiently pre-resistant subpopulations characterized by sporadic expression of resistance markers, for example alternative oncogenes such as EGFR, which can become more tolerant of a given targeted therapy. Conceptually, such 'tolerance by sporadic bypassing' is similar to the sporadic high expression of multidrug efflux pumps in bacterial populations [53]. It is currently not clear whether these pre-resistant cancer subpopulations also adopt a non/slow-growing state. Another open question is whether this mechanism constitutes a 'bug or feature' of mammalian regulatory networks: is the sporadic activation of signaling kinases merely the inevitable consequence of stochastic fluctuations within highly nonlinear signaling networks, or an evolved bet-hedging strategy? As we will discuss in the next section, recent evidence suggests that at least bacterial persistence may indeed be an evolvable trait.

3. Persistence and evolution

Given the ubiquitous cell-to-cell variability in gene expression, it is tempting to assume that persistence is an inevitable byproduct of life ('persistence as stuff happens') [54]. However, there is some evidence that bacterial persistence is actually an evolvable trait [55,56]. *E. coli* cultures exposed to different frequencies of antibiotic treatment quickly evolve an inverse relationship between persister fractions and treatment interval, without altering their antibiotic resistance [55]. This observation is of particular importance given evidence that antibiotic tolerance acts as a stepping-stone on the path to resistance. A study by Levin-Reisman et al. demonstrated in a series of *in vitro* evolution experiments that Ampicillin-resistant *E. coli* mutants emerge from the pool of initially only Amp-tolerant mutants [57]. A potential explanation for this result is that the space of mutations conferring tolerance – and therefore the probability to establish a tolerance-inducing mutation – is substantially larger than the space of mutations conferring resistance [5].

Interestingly, recent work suggests that cancer persister cells are also an important reservoir for the emergence of resistant cell lines *in vitro* [10,58]. Hata et al. focused on the clinically relevant EGFR-T790M gatekeeper mutation, which makes EGFR-mutant non-small-cell lung cancer (NSCLC) cells resistant to EGFR inhibitors. The authors found that EGFR-T790M positive populations not only originate from the selection of pre-existing mutants, but can also emerge from the pool of persister cells. Moreover, work by Ramirez et al. suggests that even within an initially isogenic EGFR-mutant NSCLC cell population exposed to an EGFR-inhibitor, the persister subpopulation ultimately gives rise to different mutant populations with diverse resistance mechanisms [58]. These results indicate that the evolution of drug resistance is not necessarily restricted towards few attainable bypass mechanisms when cell populations first pass through a persister state.

If resistant mutants indeed evolve from the pool of persister cells in a population, a rational strategy to minimize their emergence is the elimination of persisters before drug exposure. However, as we discussed above, persisters not only emerge spontaneously in untreated populations, but can also be induced by various environmental stresses. Recent *in vitro* studies have begun to elucidate the importance of induced persistence, also termed ‘type I persistence’ [9], in cancer cell populations [31,45]. Pisco et al. showed in clonally derived leukemia cells that the rapid emergence of multidrug resistance 1 (MDR1) mediated persisters upon chemotherapeutic treatment is largely driven by induced persistence [45]. Whether such ‘Larmarckian induction’ [45] is the exception or the norm in the emergence of cancer drug persisters remains an open question. Nevertheless, these works suggest that inhibition of the mechanisms that mediate the transition to a persister state during drug treatment may help to prevent the emergence of drug resistance in cancer [31,45,58–60].

4. Conclusions and open questions

In this review, we explored the surprising parallels between bacterial and cancer persisters that are emerging in recent literature. In particular, both persister types are frequently associated with a slow-growing phenotype, show metabolic alterations that leave them vulnerable to metabolic perturbations, and rely on a distinct regulatory program that can be targeted to prevent persister formation.

Currently, the mechanistic links between the emerging regulatory processes in cancer persisters, namely EMT and chromatin remodeling, their slow-growth phenotype, and their vulnerability to inhibitors of lipid peroxidation and oxidative phosphorylation [11,13,35–37], are unclear. Lessons from bacterial research may provide some inspiration. For example, recent works have shown that the global coordination of protein expression in bacteria heavily depends on the growth rate [61–65] and can be described by few so-called ‘growth laws’ [66]. It is tempting to speculate that in cancer cells similar mechanisms could potentially induce an EMT-type transcriptional program if growth is impaired. Such a mechanism might explain the rapid increase in persister fraction that has been observed in drug exposed populations [31,45]. Another intriguing question is whether metabolism can directly induce persistence in cancer cells, similar to the nutrient-shift induced persisters in bacteria [21–24]. Interestingly, recent reports suggest that loss of fumarate hydratase may induce EMT through fumarate-mediated changes in epigenetic state [67], thus providing a

potential link from metabolism to EMT-induced drug tolerance. Future efforts might identify scenarios in which metabolism sits in the driver seat of cancer persister formation.

A major aspect not discussed in this review is the influence of the microenvironment on persister formation. Bacteria living in in communities—termed biofilms—tend to have higher persister fractions than planktonic cultures [8]. Do nearby cells also affect the formation of cancer persisters, for example through direct cell contact or indirectly via signaling molecules? *In vitro* observations have shown that growth factors can attenuate the efficacy of oncogene-targeting drug therapy by activating alternative signaling pathways [68]. It is conceivable that other molecules present in the tumor microenvironment, such as cytokines or metabolites secreted by cancer cells, might also modulate persister formation. An important first step will be the systematic identification of tumor microenvironment molecules that play a role in the formation and maintenance of cancer persisters. Future experiments might also explore the extent to which cell-cell contacts among cancer cells and between cancer and stroma cells affect persister formation. Here, the self-organizing 3D structure and microenvironment provided by organoid systems may provide interesting new research avenues [69].

Finally, the relevance of persisters in clinical settings remains an open question. There is indeed evidence that antibiotic persisters play a role in bacterial infections [4]. For example, *Pseudomonas aeruginosa* strains infecting patients with cystic fibrosis show dramatically increased persister levels over time, which seems to be main mechanism to cope with antibiotic treatment [70]. There is also evidence in murine *Salmonella typhimurium* infections that slow-growing persisters survive antibiotic treatment and drive disease progression [71]. In contrast, the role of persisters in tumor progression is more enigmatic. So far, evidence is mostly restricted to mouse models [12,72]. For example, slow-growing glioblastoma subpopulations were reported to survive initial drug exposure and repopulate the tumor after cessation of drug treatment [12]. Assessing the clinical relevance of persisters could follow in two steps. First, molecular signatures that are unique for preclinical models of cancer persisters will need to be identified. The aforementioned signatures of EMT or chromatin remodeling in persisters could provide a starting point. Second, the presence of these signatures in clinical samples needs to be validated, for example through examination of serial patient biopsies throughout treatment. Ultimately, validated signatures could be used to evaluate clinical efficacy of persister-targeting therapies, including targeting metabolic vulnerabilities of cancer persisters or preventing the transition into a persister state in the first place.

Acknowledgements

KK acknowledges funding from the European Molecular Biology Organization (Longterm fellowship ALTF 1167–2016). LM is funded by a NSF Graduate Research Fellowship. This work was funded by NSF PHY-1545915 (SA), SU2C/MSKCC (SA), NCI-NIH RO1 CA185404 and CA184984 (LW) and the Institute of Computational Health Sciences (ICHS) at UCSF (SA and LW). The authors thank Nathalie Balaban, Aaron Hata, Mattia Zampieri, Jeremy Chang, Louise Heinrich, Chien-Hsiang Hsu, Laura Sanman, Alice Sebag-Schoenauer, Xiaoxiao Sun, and Christopher Waters for helpful feedback on the manuscript.

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Highlights

- Drug persistence is frequently observed in bacterial and cancer cell populations
- Slow growth, and a distinct metabolic and regulatory state are common persister hallmarks
- It is unclear how growth, metabolism, and regulation are linked mechanistically in cancer persisters
- Persisters can serve as a reservoir for the emergence of resistant mutants
- Targeting distinct persister vulnerabilities may provide new therapeutic avenues

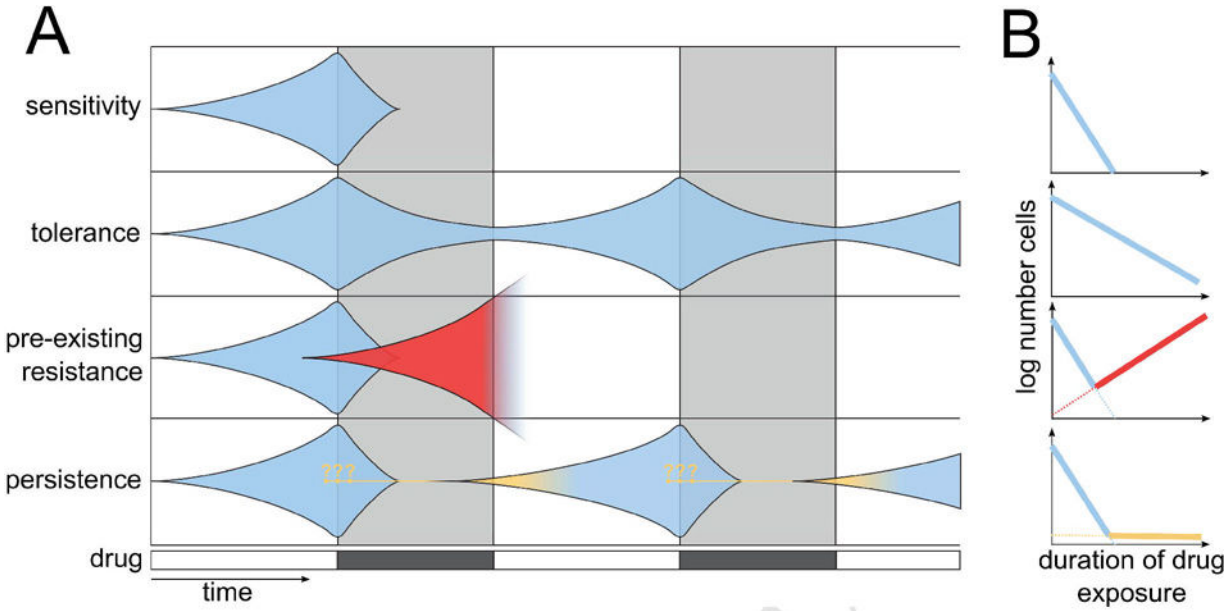

Figure 1. Schematic of different forms of drug sensitivity.

A) Blue area: Size of cell population. Grey shaded area: duration of drug treatment. Sensitivity: cells in a drug-sensitive population are readily killed by the drug. Tolerance: a drug-tolerant population is killed at a slower rate than a sensitive population. Pre-existing resistance: drug-treatment selects pre-existing resistant mutants (red), which continue growing while sensitive cells are being killed. Persistence: persister subpopulations (yellow) form either before drug treatment (type II persistence), or are induced by treatment (type I persistence), as indicated by yellow question marks, and survive the duration of drug treatment. Once treatment is stopped, persisters re-establish a mixed population of sensitive and drug-tolerant cells, which remains susceptible to repeated drug exposure. **B)** Corresponding survival curves, plotted as the log number of cells over time during drug exposure.

Table 1.

In vitro persister model systems in cancer (Sorted by cancer origin and cell line name). Corresponding references are included in brackets.

Cancer	Cell Line	Target	Drug	Susceptibility
Breast	BT474	HER2	Labatanib, Trastuzumab	BAD/BCL-XL [73]
Breast	BT474	HER2	Lapatanib, Carboplatin+Paclitaxel	GPX4 [11]
Breast	EVSA-T	PI3K	PI3 kinase inhibitor	KDM5 [46]
Breast	SKBR3	HER2	Lapatanib	KDM5 [46]
Colon	Colo205	BRAF	Vemurafenib	KDM5 [46]
Gastric	GTL-16	MET	Crizotinib, Etoposide	ALDH1A1 [36]
Gastric	MKN-4	MET	Crizotinib	ALDH1A1 [36]
Lung	HCC827	EGFR	Erlotinib	BCL-2/BCL-XL, pSTAT3 [74], SOX2 [75]
Lung	HCC827	EGFR	Gefitinib	OCT4 [76], HIF1a, IGF1R [77]
Lung	PC9	EGFR	Erlotinib	BCL-2/BCL-XL, pSTAT3 [74], KDM5 [46], GPX4 [11]
Lung	PC9	EGFR	Gefitinib	IGF-1R, KDM5 [2], OCT4 [76], HIF1a, IGF1R [77]
Ovarian	JCRB		Carboplatin+Paclitaxel	GPX4 [11]
Skin	A375	BRAF	Vemurafenib	GPX4 [11]
Skin	Hs888	BRAF	AZ628	KDM5 [46]
Skin	M14	BRAF	AZ628	KDM5 [46]
T-ALL	DND-41		GSI (Compound E)	BRD4 [78]
T-ALL	KOPT-K1		GSI (Compound E)	BRD4 [78]