Understanding Drug Sensitivity and Tackling Resistance in Cancer

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ABSTRACT

Decades of research into the molecular mechanisms of cancer and the development of novel therapeutics have yielded a number of remarkable successes. However, our ability to broadly assign effective, rationally targeted therapies in a personalized manner remains elusive for many patients, and drug resistance persists as a major problem. This is in part due to the well-documented heterogeneity of cancer, including the diversity of tumor cell lineages and cell states, the spectrum of somatic mutations, the complexity of microenvironments, and immune-suppressive features and immune repertoires, which collectively require numerous different therapeutic approaches. Here, we describe a framework to understand the types and biological causes of resistance, providing translational opportunities to tackle drug resistance by rational therapeutic strategies.

Introduction

Drug resistance as a major problem in cancer

Tumors are dynamic, evolving entities that comprise a complex ecosystem involving diverse cell populations of the tumor microenvironment, such as stromal and immune cells. Furthermore, tumors continuously react to extrinsic factors including extracellular matrix, glucose and other metabolic nutrients, growth and signaling factors, immune surveillance and selective pressure of therapeutics. Therapeutic agents can include any external treatment approach aimed to reduce the tumor cell burden including surgery, radiotherapy, chemotherapy, targeted therapy, or immunotherapy. Many tumors are successfully eradicated by a properly functioning immune system and others can be eliminated with therapeutic regimens that are cytotoxic to the tumor and/or that boost the antitumor immune response. However, complete responses in advanced tumors are rare, and far too many tumors adapt to become resistant to therapy. Thus, therapy resistance remains a significant limitation toward achieving the desired "cure" in patients with cancer. Indeed, drug resistance is believed to underlie a majority of cancer-related deaths. Attention to this problem has resulted in extensive investigations into the causes of drug resistance and possible solutions over several decades. The list of potential causes for drug resistance is lengthy and has included clonal evolution of tumors toward a resistant clone (1), increased expression and/or activity of multidrug efflux pumps (2), the elusive nature of cancer stem cells (3) and the role of shifting epigenetic states in shaping plasticity of these cells (4), microenvironmental impacts on all hallmarks of cancer (5) inclusive of changes in acidity (6), which plays a role in altered metabolic states (7) and regulation of recycling mechanism such as autophagy (8). In fact, most of these causes are mechanistically linked creating a complex and dynamic setting of chemoresistance for which effective salvage regimens have generally been elusive. Clearly, this significant unmet medical need demands novel approaches to understand drug resistance and more effective therapeutic strategies to prevent or reverse resistance mechanisms that promote tumor survival. Ideally, these new approaches will be structured upon new and granular knowledge of the biological changes that occur as tumors and tumor cells evolve under drug treatment, the way in which specific environmental pressures can select for specific tumor features, and the shifting of broad cellular states that can be coordinated with tumor molecular features.

Tumor heterogeneity is a significant factor contributing to drug resistance

Immense heterogeneity is an inherent feature underlying the diverse molecular, cellular, tumor-intrinsic, and tumor-extrinsic biology within the tumor ecosystem. Intratumor and intertumor heterogeneity can be described by multiple mechanisms, such as those detailed further in this review. These mechanisms participate in the Darwinian theory of branching evolution resulting in the formation of the fittest clones with

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heritable characteristics capable of long-term survival in response to their tumor environment and drug exposure. Therapeutic treatment of tumor clones represents one agent for the selection of acquired drug-resistant populations within the tumor ecosystem (9). Similarly, a transient, nonheritable selection of drug-tolerant cells based on rare cell variability has been described in a Lamarckian-like evolution model of drug resistance in cancer (10–12). Different to Darwinian selection, Lamarckian induction refers to the acquisition of favorable features in response to selective pressure in a nonhereditary fashion (13). Of note, the evolutionary concepts described in this review are global and resemble, to some degree, antimicrobial resistance in bacteria (14). However, unlike the treatment of simpler microbial organisms, cancer represents a more complex ecosystem with the inherent challenge of sharing essential characteristics with benign cells from which tumors are derived. Rational approaches and novel strategies such as evolutionary game theory (15) will be necessary to understand and overcome treatment resistance.

Types of Resistance

Responses to rationally targeted therapies can be divided into several different forms based on depth and duration of response as well as mechanisms of eventual resistance. Broadly speaking, these treatment responses can be classified into three categories (**Fig. 1A**).

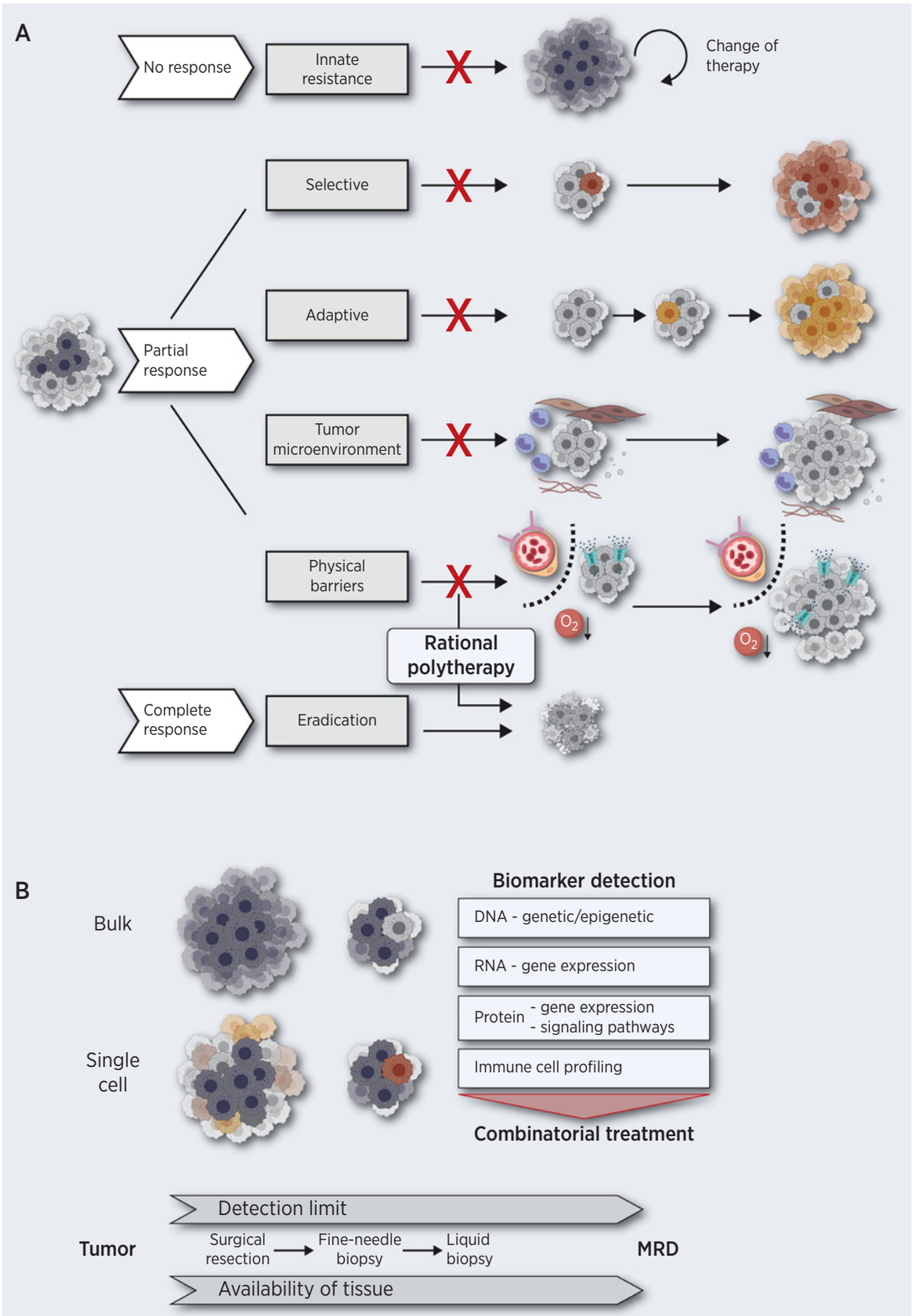
Resistance lacking a notable response

Also referred to as innate resistance or primary refractory, this scenario results from a rapid treatment relapse and continued outgrowth of cancer cells under drug treatment. It is driven by the presence of tumor features in the bulk tumor cell population that confer insensitivity to the therapeutic agent or regimen. This includes the bulk tumor harboring a single or a constellation of cooccurring mutations that result in drug resistance (16, 17). As outlined in later sections of this review, resistance-associated mutations can include target-intrinsic, pathway-intrinsic as well as broader bypass tumor-intrinsic alterations. Target-intrinsic mutations causing resistance are exemplified by EGFR T790M and C797S mutations in non–small cell lung cancer (NSCLC) mediating resistance to EGFR inhibitors erlotinib and osimertinib, respectively (18–20), as well as by PSMB5 mutations mediating resistance to bortezomib and other proteasome inhibitors in multiple myeloma (21). Pathway-intrinsic alterations or bypass mutations are represented by MEK1/MEK2 mutations, MITF amplification, PIK3CA mutations, and PTEN loss in BRAF-mutant cancer which confer resistance to RAF inhibitors (22, 23) as well as MET amplification and CDK4/6 amplification in EGFR-mutant NSCLC which confer resistance to EGFR inhibitors (24, 25). Similarly, splicing dysregulation can hamper drug response to targeted therapy or increase disease aggressiveness. As such, cooccurring mutations in splice factor RBM10 have been reported to limit therapeutic response to targeted therapy due to alternative splicing of BCL2L1 reducing drug-induced apoptosis in EGFR-mutant NSCLC (26). In prostate cancer, splicing dysregulation correlates with disease progression (27), with splice factor SRRM4 being involved in alternative splicing of LSD1 (KDM1A) and contributing to the expression of genes linked to aggressiveness in neuroendocrine prostate cancer (28). Given the dominance of intratumor heterogeneity, multiple bypass mutations may occur within the same tumor and drive polyclonal drug resistance (16). Furthermore, alternative features such as gene expression patterns or cell differentiation states can result in poor efficacy of therapeutic tumor cell killing and may cause innate resistance when affecting the bulk tumor population (29–32). This may include treatment-induced transdifferentiation of tumor cells to an alternative, drug-resistant cell lineage/state causing treatment resistance. The latter is exemplified for the transdifferentiation of advanced EGFR-mutant lung adenocarcinoma to small cell lung cancer (33), transdifferentiation of AR-active prostate adenocarcinoma to an AR-null small cell neuroendocrine carcinoma (34, 35) as well as cellular plasticity of melanoma cells to a neural crest stem cell–like state (36). Similarly, drug efficacy can be nullified by rapid feedback activation of oncogenic signaling, as exemplified by the limited treatment response of KRAS-mutant tumors to single-agent KRAS (37) or MEK inhibitors (38) or of estrogen receptor–positive breast cancer to single-agent PI3K inhibition (39).

Partial response with residual tumor cells remaining after initial treatment

Partial response is characterized by an initial drug-mediated reduction of tumor growth, followed by stagnation of treatment response and the selection or adaptation events that allow drug-tolerant tumor cells to remain and ultimately to drive the evolution to resistance (40). Tumor heterogeneity and the presence of treatment-resistant or tolerant subpopulations at a low cellular frequency can result in residual tumor cell lesions (36). Indeed, target-intrinsic or pathway-intrinsic alterations observed for partial response driven by a selection of cancer cell clones may overlap with mechanisms outlined above but affect a significantly smaller fraction of tumor cells in partial response specimens, while the bulk tumor is treatment sensitive in this scenario. As such, mutational heterogeneity across tumor cell lesions is associated with differential depth of treatment response and the subclonal presence of alternative oncogenic drivers has been observed at a low cellular frequency (24, 41). Similarly, secondary mutations in the drug target may be found in a small subpopulation of cancer cells and mediate the selection of resistant tumor cells after an initial treatment response (10). In addition to selection based on genetic alterations, transcriptional variability at a single-cell level can result in a transient state of higher expression for survival-promoting proteins and may mediate the selection of drug-tolerant cells under treatment (11). As such, expression of plasma cell maturation markers in a small subpopulation of multiple myeloma cells (0.1%–12%) is indicative of the development of acquired resistance to proteasome inhibitors and reflects an overlapping feature with innate resistance if present at a clonal level (29). Overexpression of proteasome subunits (42) or loss of surface expression of the drug target (43) may similarly mediate resistance development to proteasome inhibitors and anti-CD38 mAbs, respectively, in multiple myeloma and similar concepts are observed in other cancer entities (44).

Alternatively, drug tolerance can be driven by adaptive, nongenetic mechanisms such as transcriptional and epigenetic alterations as well as metabolic rewiring (45, 46). Changes in the methylation of DNA and histones as well as altered transcription factor activity may promote the transition to favorable cell states that allow a small subpopulation of cells to withstand drug treatment. Such cell states most often comprehend broader tumor-intrinsic mechanisms that promote resistance, as outlined in later sections of this review. Among others, changes in histone methylation have been reported to regulate the expression of cell cycle–associated genes and drive drug tolerance in NSCLC (47). Similarly, the engagement of YAP/TAZ transcription factors mediates the expression of antiapoptotic and mesenchymal cell state [epithelial–mesenchymal transition (EMT)]-associated genes in drug tolerance and resistance in NSCLC and melanoma (48, 49). These data have opened up a new therapeutic avenue to disrupt YAP-TEAD interactions (48, 50). In addition, deregulated YAP signaling which



activates antiapoptotic machinery has been shown to create new vulnerability for ferroptosis inducers (51). In breast cancer, EMT and subsequent FGFR activation are central to tumor regrowth from minimal residual disease (MRD; ref. 52). Transitioning into a state of slower proliferation and associated alterations in DNA repair processes have been shown in drug-tolerant cells (53, 54) and may contribute to the subsequent genetic evolution of drug-tolerant persister cells to fully resistant cells (10, 55). Furthermore, activation of growth factor receptors and hormonal receptors is a common adaptive response observed in persister cells. In melanoma, activation of ERBB2/ERBB3 and EGFR has been observed in response to MEK inhibitors and BRAF inhibitors, respectively (56, 57). In prostate cancer, castration induces upregulation of androgen receptor (AR) expression (58), and activation of the glucocorticoid receptor has been shown to bypass AR inhibition by next-generation anti-AR agents like enzalutamide (59). In parallel to outlined transcriptional changes affecting cell cycle and EMT in residual drug-tolerant cells, adaptations of cellular metabolism marked by increased mitochondrial respiration in drug-tolerant persister cells (60, 61) induce elevated oxidative stress as well as dependency on glutathione-dependent redox metabolism, for example, via GPX4 (62) or NRF2 (63). Furthermore, drug-tolerant persister cells are characterized by the evasion of drug-induced apoptosis, which may partly be mediated by YAP-driven expression of antiapoptotic BCL2L1 (49) as well as increased levels of antiapoptotic MCL1 upon drug treatment (64). Adaptive activation of inflammatory NFκB, IFNγ, and STAT3 signaling may further hamper drug response and represents a common feedback node across cancers harboring different oncogenic driver gene mutations (65–67).

In addition to tumor cell–intrinsic adaptation, tumor-supportive interactions with cells of the tumor microenvironment can drive the survival of cancer cells under drug treatment. As such, tumor-associated macrophages, cancer-associated fibroblasts (CAF), and protumorigenic subsets of T and B lymphocytes promote drug tolerance by secreting cytokines, chemokines, small RNAs, and metabolites, while also lacking or actively inhibiting a functional immune response against cancer cells (68, 69). Of note, treatment-induced senescence and the associated senescence-associated phenotype may critically contribute to the formation of a tumor supportive niche given the dominant cytokine cross-talk (70). Secretion of hepatocyte growth factor (HGF) by CAFs and its subsequent stimulation of MET on cancer cells exemplifies the prosurvival cross-talk with noncancer bystander cells in drug resistance (71) and may further inhibit T-cell expansion and effector functions (72). Similarly, cancer cells educate macrophages towards a tumor-supportive M2 phenotype (73), as exemplified by the tumor cell–mediated secretion of exosomes stimulating the expression of immune checkpoint molecules on myeloid cells (74). Expression of immune checkpoint molecules, such as PD1/CD274 (PD-L1) and CTLA4, limits T cell–driven cytotoxicity (75) and an increased macrophage infiltration correlates with poor disease outcome across different cancer entities (73, 76). Furthermore, tumor-associated macrophages are characterized by their secretion of IL6, HGF, and other soluble factors that activate STAT3 signaling, prosurvival pathways, and metabolic rewiring in tumor cells (77–79). In glioblastoma, cell-cell crosstalk between tumor cells and macrophages further promotes a mesenchymal cell state in cancer cells and is associated with T-cell infiltration (80), pointing to a differential role of macrophages within the tumor microenvironment. Adding to this, recent work incorporating spatial exome and RNA sequencing with machine learning–based spatial histology has shown the immune geospatial variability within tumors and pointed to the selection of immune-evading cancer cell subclones (81)—highlighting the relevance of previously described tumor heterogeneity in the context of tumor microenvironment interactions and cancer immunoediting. Of note, advances in the targeted treatment of hematologic malignancies have further demonstrated the impact of reducing tumor cell interactions with their supportive microenvironmental niche, as both ibrutinib treatment in chronic lymphocytic leukemia as well as anti-CD38 treatment in multiple myeloma reduces the contact of cancer cells with their tumor microenvironment niche, which contributes to increased therapeutic efficacy and the priming of additional vulnerabilities (43, 82).

Finally, physical barriers may inhibit complete drug responses as highlighted by the lack of permeability for targeted inhibitors across the blood–brain barrier (BBB; refs. 83, 84) or the disorganization of vascularization in fast-growing tumors (85), both limiting efficacious doses at the tumor site. Of note, the tumor microenvironment is critically involved in mediating an angiogenic switch in tumors, with tumor-associated macrophages and CAFs promoting angiogenesis and contributing to the disposition and organization of the extracellular matrix (ECM) in tumors (86). The latter is forming a barrier for the access of therapeutic agents to tumor cells within their tissue niche (87). In addition, alterations in the expression of

Figure 1.

Molecular differences in the response to targeted therapies and their characterization using different technology platforms. **A,** Types of resistance. Responses to targeted therapies can be classified as (i) resistance lacking a notable response, (ii) partial response with residual tumor cells remaining after initial treatment, and (iii) complete response showing a full eradication of the tumor. Innate resistance is characterized by a rapid treatment relapse and continued outgrowth of cancer cells under drug treatment. It is driven by the presence of cells harboring resistance-associated mutations or their switching of cellular identity by transdifferentiation to a resistant lineage. Partial response is characterized by an initial drug-mediated reduction of tumor growth. However, residual tumor lesions remain and ultimately evolve to become drug resistant. Multiple mechanisms can mediate resistance development following initial response. Briefly, the selection of residual tumor cells harboring additional mutations, the adaptation of residual tumor cells by epigenetic, transcriptional, metabolic, or inflammatory mechanisms, the survival-promoting input from a tumor-supportive microenvironment, and cell or tissue level physical barriers may hamper therapeutic efficacy in partial response. Advances in drug resistance mechanism research are required to define both common and unique targetable nodes across cancer entities, based on which rational combinational therapies can be identified to inhibit the development of resistance and deepen treatment response. **B,** Defining biomarkers and mechanisms using high technology platforms. Recent advances in the profiling of clinical specimens have improved patient stratification into oncogene- or epigenetically defined cancer subsets. Profiling by bulk whole-exome sequencing or RNA sequencing as well as the targeted analysis for the expression of oncogenic drivers allow important insight into the presence of a clonal driver and respective treatment decisions. Profiling of cancer specimens on a single cell level, such as by single-cell RNA sequencing, allows additional depth characterizing tumor heterogeneity and the detection of subclonal differences regarding the mutational or transcriptional/epigenetic landscape. Different clonal sizes and their dominance in samples taken longitudinally after treatment initiation became apparent and thus, sequential evolution versus subclonal presence may be distinguished and treatment adaptations are possible. Cell-free technologies, such as liquid biopsies based on cell-free DNA analysis from blood or cerebrospinal fluid samples, may allow additional resolution of treatment-resistant cancer cell clones in a timely manner when tissue access is limited. However, despite advances in the resolution of mutational and transcriptional heterogeneity, availability of clinical specimens and technical restrictions regarding the detection limit present challenges regarding the identification of MRD at the primary or secondary tissue sites. Created with BioRender.com.

drug-metabolizing enzymes, drug transport channels, or drug efflux pumps may reduce the drug level in cancer cells and are highlighted in later sections of this review.

Complete response showing a full eradication of the tumor

Complete response or remission is characterized by a disappearance of all cancer lesions and normalization of other disease markers, indicating clearance of the tumor. While molecular targeted therapy shows significantly improved clinical efficacy and safety, complete responses in advanced-stage cancers are rare (40). Thus, rational combinatorial treatment options that address mechanisms of drug resistance or tolerance are needed to improve patient survival and ultimately cure cancer.

Biomarker efforts to enable precision medicine in cancer drug resistance

Molecular targeted therapy is tightly linked to a detailed characterization of the available clinical specimens and the identification of biomarkers (**Fig. 1B**). In residual disease and resistance, the early detection of resistant tumor cell clones is crucial for clinical decisions regarding combinatorial treatment regimens and the upfront eradication of residual tumor cells.

Advances in sequencing techniques have enabled a systematic identification of somatic mutations and epigenetic alterations within the bulk tumor that underly cancer initiation and progression, forming the basis of personalized cancer medicine. Individual cancer entities can be subgrouped according to different oncogenic driver mutations or epigenetic subsets, as highlighted for breast cancer, melanoma, NSCLC, and others (88–92). Furthermore, cross-cancer evaluations spanning different cancer entities identified reoccurring pathway alterations across RTK-RAS-MAPK, PI3K, cell cycle, TP53, Notch, MYC, Hippo, WNT, TGFB1, and NRF2 signaling pathways (93). In addition to classical DNA and RNA sequencing, novel screening approaches such as protein profiling, quantitative immunoassays, and *ex vivo* drug screening are emerging and contribute to biomarker profiling efforts (94, 95). Combinational genomic profiling, computational modeling, *ex vivo* chemosensitivity assays, and evaluation of clinical data have been used to characterize sensitivity to BRD and BET inhibitors in acute myeloid leukemia (AML; refs. 96, 97).

Spatial and temporal heterogeneity drives tumor evolution and both parallel and sequential development of tumor subclones is possible (98, 99). Intratumor heterogeneity is particularly important for the selection of more resilient tumor cells under therapeutic pressure (36, 46). In addition, supportive stimuli from the tumor microenvironment can favor cancer cell survival and mediate a transition across different cancer cell states (81, 100). Profiling of tumor specimens at single-cell resolution has deepened our insight into the complexity of cancers. Analysis of spatial information using methods such as: multiplex immunofluorescence, spatial transcript profiling, multi-omic analysis with both chromatin accessibility and transcript profiling in the same cell, and imaging mass spectrometry has provided valuable insights about tumor heterogeneity (101–105). As such, recent work profiling residual disease specimens has highlighted a differential alveolar type 1 (AT1)/AT2 hybrid state of tumor cells that withstand active therapy in NSCLC and point to dynamic microenvironment changes (41). Similarly, the analysis of metastatic melanoma samples by single-cell RNA sequencing identified heterogeneous cell states across tumor cells as well as tumor-infiltrating immune cells (106). These data on the complex architecture of tumors have opened an important new area of investigation on the cell of origin that promotes drug resistance (107). Lineage tracing methods have given deeper insights on tumor evolution, and work to date suggest selection of specific subpopulation with late-hybrid EMT states may occur during pancreatic cancer progression (108, 109).

Given the low frequency of drug-tolerant or -resistant tumor cells within a treatment-sensitive bulk tumor population, additional depth as well as innovative clinical protocols are needed to profile biomarkers of residual disease and treatment resistance. In hematologic malignancies, the sensitivity of detection methods for the assessment of MRD has significantly increased, with the detection of MRD in clinical specimens negatively impacting treatment outcomes for patients with cancer (110, 111). In solid tumors, liquid biopsies allow the monitoring of tumor evolution under therapy when tissue specimens are not available and partly overcome limitations regarding the detection of lesion-specific alterations (25, 112–114). Across different cancer types, longitudinal sampling of cell-free DNA in bodily fluids showed dynamic changes in nonsynonymous mutations, with resistance-associated mutations appearing during the course of treatment and contributing to the formation of metastatic tumors (25, 114).

Biological Causes of Resistance

A diversity of biological mechanisms can facilitate tumor cell escape from therapeutic pressure and the capacity of cancer cells to persist in a drug-resistant state (**Fig. 2**). These mechanisms range from a single-nucleotide/amino acid substitution that preclude a drug from effectively binding its target to whole state changes of tumor cells that facilitate evasion of a drug's activity or dependence on the drug target. Additional mechanisms can include rescue signals from cell–cell interactions and can involve whole organ architecture.

Many of these biological mechanisms are centered around the capacity of a target or pathway to remain active after exposure to a drug and/or adaptive processes that occur after the drug has reached its target. However, prior to discussing these categories of drug resistance, it is important to include a summary of other mechanisms that prevent a drug from adequately reaching its target. Indeed, some of the best-studied mechanisms of drug resistance involve mechanisms that actively or passively limit adequate drug availability within target cells. Both, systemic and cellular barriers have been reported and an outline of key mechanisms is presented below:

Drug access

Systemic barriers

Systemic barriers are encountered by drugs even before the drug reaches the target tissue. A major determinant for the efficacy of drugs targeting cancer cells is the BBB or blood–tumor barrier (BTB). Specifically, BBB penetrance has been a challenge in designing drugs targeting brain tumors like glioma (83). Furthermore, brain metastases of malignancies such as breast cancer have been shown to have compromised BTB in metastatic lesions, resulting in poor drug penetrance to the metastases (84). More recently, peptides that naturally cross the BBB have been used in conjugation with drugs to assist in efficient drug penetration (115). Similarly, cell-derived extracellular vesicles or synthetic liposomes represent novel nanocarrier strategies to deliver therapeutic agents across the BBB (116).

Metabolic barrier

In addition to the systemic barriers, metabolic barriers play a role in determining the concentration of active drug reaching the target. Expression levels of xenobiotic metabolic enzymes of the cytochrome P450 (CYP) family and UDT glucuronosyltransferase family in tumors as well as in the surrounding tissues can modulate the sensitivity of

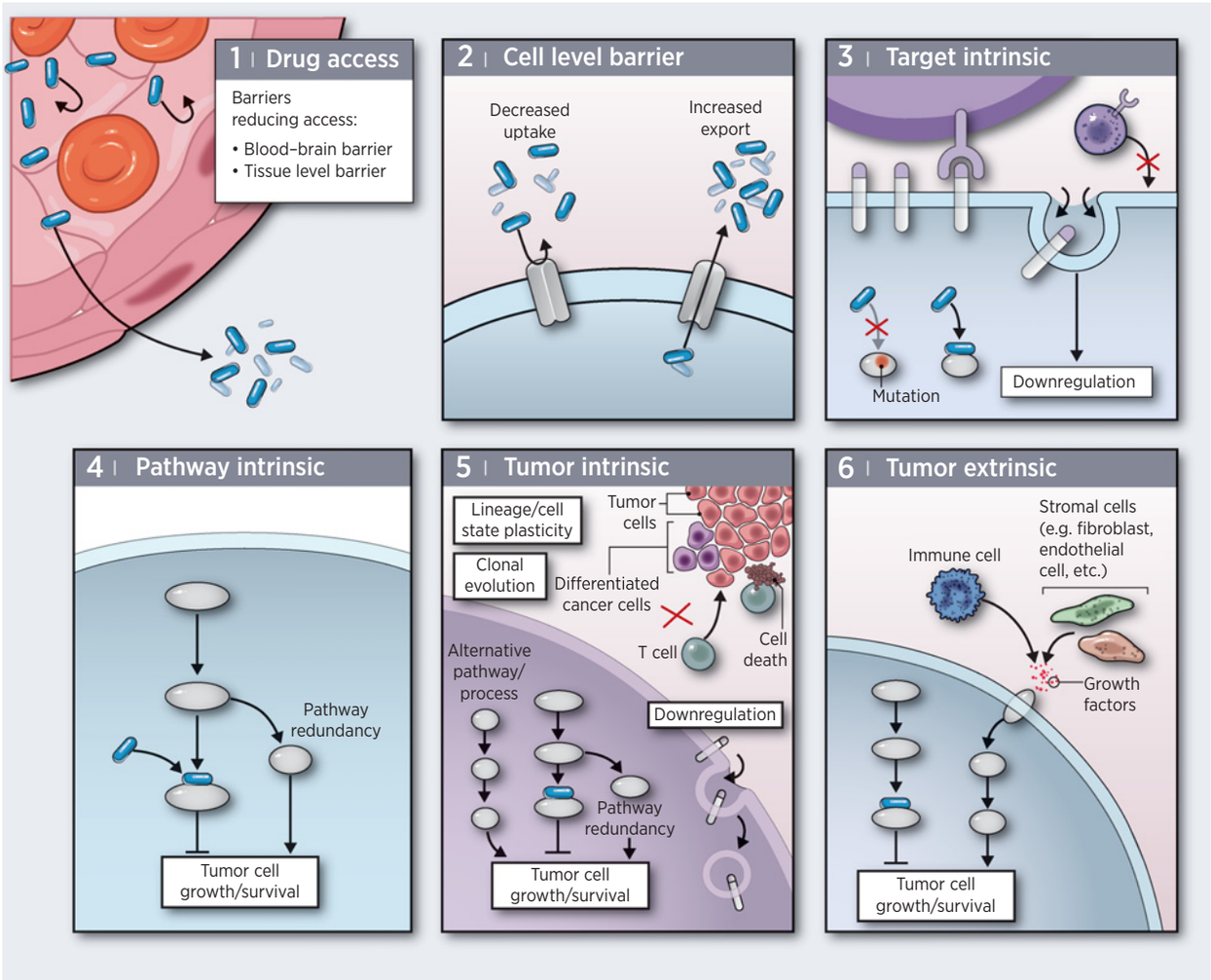


Figure 2.

Biological causes of resistance. Resistance-causing mechanisms can be classified into distinct subgroups, including (1) systemic barriers limiting drug access, (2) cell level barriers hampering intracellular drug levels and efficacy, (3) mutations or downregulation of the direct drug target, (4) alternative mutations downstream or orthogonal to the drug targets reestablishing the targeted pathway, (5) broader alterations in bypass pathways, transcriptional programs and cell lineages causing tumor intrinsic resistance and clonal evolution under treatment, and (6) tumor-supportive extrinsic mechanisms from the tumor microenvironment.

cancer cells to chemotherapeutic drugs (117, 118). Upregulation of CYP family members conferring resistance to specific anticancer drugs has been reported in breast (119), colorectal (120), ovarian (121), and renal carcinomas (122), among others (123).

Cell level barrier

Even after a drug effectively bypasses systemic barriers, there are still cell level barriers that may impact drug entry or retention. Not surprisingly, these cell level barriers are highly regulated. Most drug molecules are transported inside cells through solute carrier (SLC) transporters (124). Because cancer cells depend on SLC for nutrient uptake, the expression profile of SLCs varies across tumor types depending on their preferred energy source (124). Furthermore, cancer cells have been shown to alter the expression of SLCs in response to a drug, thereby, impairing the effective uptake of drugs such as chemotherapeutic agents (124, 125).

Similarly, hurdles in drug retention are impacting treatment efficacy after a drug has entered the cell. Drug efflux pumps, including the ATP-binding cassette (ABC) transporters, play a key role in determining drug retention rates within cells (126). Upregulation of ABC transporters like Breast Cancer Resistance Protein (BCRP also known as ABCG2) has been shown to confer resistance to cytotoxic drugs, including flavopiridol, irinotecan, methotrexate, mitoxantrone, and topotecan (126) in multiple cancers (127). In prostate cancer cells, ABCB1 causes efflux of the chemotherapeutic agent docetaxel, one of the principal treatments for the disease, and targeting ABCB1 efflux resensitized prostate cancer cells to chemotherapy (128). Emerging data from studies characterizing interaction between ABC transporters and tyrosine kinase inhibitors (TKI) suggest that TKIs can act as substrate or inhibitors of ABC transporters (129, 130). TKIs have been shown to restore sensitivity of NSCLC cells with overexpression of ABC transporters such as ABCB1 and ABCG2 to

chemotherapy suggesting that TKIs may be used as chemotherapy sensitizers (131, 132).

Taking these important drug access mechanisms of resistance into account, it is critical to properly evaluate pharmacokinetics and pharmacodynamics of investigational agents when considering potential causes for lack of efficacy. If the therapeutic agent(s) are not effectively reaching the impacted organ, are not reaching threshold levels in the whole tumor, and/or are not reaching high enough concentrations for a long enough duration inside the tumor cells, then there is no need for further adaptive or selective processes by the tumor to evade therapeutic pressure. Assessment of pharmacokinetics/pharmacodynamics is, therefore, critically important to build into preclinical models and clinical trials. Provided that these pharmacokinetic/pharmacodynamic measurements do indicate drug is reaching target at sufficient levels, then there are a number of additional mechanisms for drug resistance to consider.

Target-intrinsic mechanisms

The simplest mechanism by which tumors evade a drug that is actively reaching the target is selection for (or acquisition of) a pathogenic variant in contact sites of the target that prevent the drug from effectively binding. Many of these variants may be achieved with the help of APOBEC enzymes and other mutagenic mechanisms that result in genomic instability such as chromothripsis and chromoplexy (54, 133). There are numerous examples of resistance due to secondary mutations in drug targets that preclude effective drug activity. Examples of this mechanism have been described for initial generations of antineoplastic drugs with variants in genes such as β-tubulin observed in the context of antimicrotubular drugs (134). Given the advancement of molecular targeted therapies, more recent examples have often involved kinases, such as BCR-ABL (135), FLT3 (136), KIT (137), BTK (138, 139), EGFR (18–20), HER2 (140), and ALK (141) as well as nuclear hormones receptors, including AR (142) and estrogen receptor (143). Even more recently, the diversification of small-molecule "targeted" therapies that are approved in cancer has led to target intrinsic escape mutants in other gene families as well, including BCL2 (144) and IDH1/2 (145). Target intrinsic resistance has also been described for surface antigens that are targets for antibody-based or cellular therapies, such as CD19 in acute lymphoblastic leukemia, where resistance may be accomplished by downregulating surface expression, either through the selection of cells lacking surface expression or an adaptive process of downregulating that surface target (146).

Pathway-intrinsic mechanisms

While target-intrinsic resistance is one way to maintain an oncogenic signal or process in the face of therapeutic pressure, this same phenomenon may also occur via mutation or alteration of a different node in the pathway or process that renders the drug target dispensable. Numerous examples exist of resistance to receptor TKIs due to tumor clones harboring downstream mutations in the RAS-RAF-MAPK pathway, which obviates or reduces the need for the upstream mutant kinase signal. This is exemplified by KRAS and BRAF mutations or NF1 loss in EGFR-mutant NSCLC (25, 147). Additional examples of pathway intrinsic drug resistance have been described in melanoma, where resistance to inhibitors targeting mutant BRAF can occur due to activation of the orthogonal CRAF pathway (148) or downstream activation of MEK1/MEK2 (22). In chronic lymphocytic leukemia, treatment with inhibitors of the B-cell receptor signaling pathway (e.g., BTK) has yielded drug resistance due to clones with mutations in the downstream phospholipase C gene (PLCG2; ref. 139). Similarly, resistance to IDH inhibitors may occur due to isotype switching where a tumor with prior dependence on one of the two mutant IDH isoforms switches to a clone with the dominant expression of the other mutant IDH isoform (149). In prostate cancer, androgen ligand-independent splice variants of the AR are commonly induced after treatment with novel and more potent AR inhibitors (150). The presence of these splice variants, including AR-V7, in circulating tumor cells from patients progressing on novel AR inhibitors is associated with a low likelihood of response to treatment with additional AR inhibitors that block AR's ligand-dependent function (151). Preclinical studies in AR inhibitor–resistant cell lines suggest these AR splice variants may help maintain AR signaling despite a low androgen environment (152). Moreover, recent work demonstrates that the glucocorticoid receptor is epigenetically induced after AR inhibitor treatment in some cell line models and may compensate for the AR to maintain AR signaling (59).

Tumor-intrinsic mechanisms

Drug resistance may occur as a consequence of broader changes in tumors and tumor cells. This may include a multitude of mechanisms such as clonal evolution, lineage plasticity, mesenchymal or stem cell–like differentiation states, chromatin and transcriptional adaptation, metabolic rewiring as well as autocrine and cell intrinsic inflammatory signaling. Clonal evolution and lineage plasticity in therapy resistance has been well documented in multiple cancers (41, 153–156). In part, nodes of clonal evolution may overlap with pathway intrinsic resistance, for instance in IDH- or FLT3-mutant cancers exhibiting resistance through the selection of clones that harbor additional or alternative mutational profiles, such as mutations of RAS pathway genes (155–157). Other examples exist where the selected clones do not replicate the originally active pathway but, instead, present an entirely different pathway or process that is dysregulated. In some cases, this process may select or drive cells into specific differentiation states, as observed for AML response and resistance to BCL2 inhibition (158–161). Common concepts of cells entering alternate differentiation states that mediate drug resistance include drug-tolerant persister cells and/or cancer "stem cells"—with overlapping features across both phenotypes (47, 162). Drug-tolerant persister cells are defined as a small subpopulation of cancer cells that withstand high-dose drug treatment by going into a state of low-to-no proliferation and avoiding drug-induced apoptosis (10, 47). They present a mesenchymal cell state and show an engagement of alternate growth factor receptors, as outlined in prior sections of this review. Similarly, cancer "stem cells" are characterized as cells of a primitive, "stem-like" differentiation state that mediates tumor resistance. In line with the latter, profiling of prostate tumor biopsies identified that reduced androgen signaling coupled with a stemness program conferred resistance to AR-targeted therapy and poor outcome in patients with prostate cancer (32). Therapeutic pressure in such complex tumors is only capable of eliminating the more differentiated tumor cells, leaving drug-resistant cancer "stem cells" intact or promoting the acquisition of a mesenchymal phenotype in drug-tolerant persister cells. Many of the traditionally used antineoplastics have conformed to this pattern; however, some recent drugs have exhibited better efficacy against the more stem-like cell populations and less activity against more differentiated tumor cell populations, causing the opposite drug resistance pattern where disease relapse is associated with persistent cells of a more differentiated state. These recent examples may merit a rethinking of the concept of tumor collapse from targeting the stem-like cell populations because it appears that self-renewal and proliferation may not require these stem-like properties. Related observations have

shown that tumor cells may exhibit lineage plasticity with resistance occurring due to transdifferentiation of tumors, for example from an epithelial to a neuroendocrine cell state in prostate cancer after AR-directed therapy (163) or, as noted previously, for lineage switching in EGFR-mutant NSCLC (33) and melanoma (36). Another form of lineage switching has been described in response to immunotherapies directed against lineage-specific antigens, for example, lymphoid leukemia cells switching to a myeloid phenotype in the context of CD19-directed CAR-T cell therapy (164, 165). The molecular determinants that govern many of these tumor intrinsic mechanisms of drug resistance may frequently differ from those that allow for target- and pathway intrinsic resistance. The latter are often facilitated by genomic changes (e.g., point mutations), while many of these tumor intrinsic mechanisms are driven by broader alterations in the tumor/tumor cell epigenetic landscape. In drug persistence, redistribution of histone H3K4 and H3K27 methylation by the activation of histone demethylases KDM5A/B and KDM6A/B, respectively, has been observed across glioblastoma (166), melanoma (61), and NSCLC (47) cancer entities, with relevance for the reduction in expression of cell-cycle genes (47). Similarly, engagement of YAP/TEAD, β-catenin as well as c-Jun/AP-1 transcription factors is involved in modulating gene expression and acquisition of mesenchymal characteristics (EMT) in drug-tolerant persister cells (41, 48, 167). In addition, loss of CHD1 results in global alterations in chromatin state leading to activation of diverse transcription factors, thereby conferring resistance to AR-targeted therapy (168).

Tumor-extrinsic mechanisms

The interactions between tumor cells and their tumor microenvironment are crucial to determining response and, sometimes, driving resistance. Indeed, some of the processes described above as "tumor intrinsic" can be influenced by signals from the microenvironment, such as epigenetic and cell state changes that can occur in response to paracrine signals from immune or stromal cells (80, 100). These signals can be in the form of secreted factors or cell-cell contact and provide growth/survival signals that permit tumor cells to persist even with an oncogenic pathway blocked by drug activity. Furthermore, examples of this can be found in myeloid malignancies where paracrine growth factor signaling permits tumor survival and changes in tumor cell state and metabolism during early stages of drug resistance that leads to eventual relapse with changes in tumor genetics (79, 169–174). Intriguingly, therapy-induced cancer cell death is recently shown to associate with the extracellular release of mitogens or damage associated molecular patterns, which correspondingly impact repopulation of residual surviving cancer stem cells (175) and immunogenic cell death—a mode of cell death that activate innate immune cells to recruit adaptive immune effectors (e.g., $CD8^+$ T cells) to augment therapy response (176). In addition, the interactions between a tumor and its microenvironment are critical mediators dictating response versus resistance to a variety of immune-based therapies including antibodies that block immune checkpoint inhibitors. A complex and bidirectional dynamic between tumor, immune, and stromal cells regulates the extent of immune suppression at baseline as well as the capacity of biologics to elicit a rescued immune response. Thus, resistance to immune checkpoint blockade has been observed to occur due to a multitude of mechanisms including, among others, (i) altered checkpoint ligand/receptor expression, (ii) changes in antigen presentation, such as loss of β2-microglobulin affecting antigen presentation, (iii) suppression of immune-stimulating pathways, such as IFNγ, JAK1, and JAK2 signaling, (iv) dysfunction of cells that are required to facilitate T-cell responses (e.g., dendritic cells, macrophages), (v) T-cell inhibition (e.g., regulatory T cells, tumor-associated macrophages) or T-cell exhaustion, and (vi) microbiome influences (177–181). Furthermore, distinct cancer-immune phenotypes are apparent, including "immune deserts" lacking reactive immune cell infiltration or antigen priming/presentation (178). Chromatin remodeling has been implicated in contributing to some of the above mechanisms, such as altered antigen presentation and suppression of proinflammatory cytokine signaling (177, 178). Similarly, dysfunctional cancer-immune phenotypes can be composed of immune cells that inhibit the normal role of the immune system to control tumor growth, such as regulatory T cells, myeloid-derived suppressor cells, and tumor-associated macrophages (178). Combination approaches that mitigate these mechanisms as well as using multiple checkpoint inhibitors may be effective against immune checkpoint therapy resistance (177, 179, 182, 183).

Cancer metastasis

There are critical challenges in the prevention and treatment of cancer metastasis, which is the major cause of death in most patients with cancer (184). Metastasis formation is a complex sequential process requiring tumor cell-intrinsic alterations such as EMT and tumor cell extravasation as well as changes in the tumor microenvironment such as premetastatic niche formation and a supportive fibroblast microenvironment, ECM remodeling, angiogenesis, and the recruitment of innate immune cells and T cells (184, 185). Most importantly, dormant cancer cells can reside within the bone marrow microenvironment and may result in metastatic relapse even after the removal of the primary tumor (184, 186). Thus, targeting metastatic colonization and the metastatic cell reservoir in the bone marrow microenvironment remain critical aspects of successful cancer therapies. As outlined by Steeg (184), targeting metastasis formation is hindered by the complex interplay of multiple processes during metastatic colonization and may require the continuous combination of different drug classes. Furthermore, the cytostatic nature of metastasis-preventing therapy may limit clinical establishment.

The Path Forward

Promises and challenges

Significant advances have been made in combatting drug resistance in cancer, which have been facilitated in a number of important ways. New technologies and analytics for biological exploration, such as deep sequencing, single-cell methods, functional readouts of primary tumor cells, and genome-wide screening approaches have revealed many new facets of cancer biology as well as the way in which cancers can respond to therapies and evolve after therapeutic exposure. These improvements in our conceptual understanding of mechanisms governing drug response and resistance have led to the deployment of numerous successful therapies and, increasingly, combination therapies that are rationally implemented on the basis of tumor biology and have led to some improvements in patient outcomes. In addition, the development of new and optimized classes of therapeutic agents has been an important contributor to the cutting edge of cancer investigation and therapy. Indeed, these innovations have collectively led to major successes in the creation and implementation of second- and third-line drugs that combat target-intrinsic resistance, drug combinations to target pathway- and tumor-intrinsic resistance, and conceptualization of combinations to simultaneously target tumor intrinsic biology as well as tumor-extrinsic resistance mechanisms.

Despite these significant achievements, notable obstacles remain. Some of these challenges have been unveiled by the very same technologies and studies that yielded the above successes. Tumor heterogeneity, while always a recognized feature of tumor biology, has been uncovered with sharp relief using single-cell functional, proteomic, and deep sequencing techniques. This intratumoral complexity yields an incredible matrix of combinatorial features that tumors can display with resistance mechanisms sometimes already lurking in tumor subpopulations and sometimes adaptively developing in response to therapy. An important question that will be important to tackle in the coming years relates to these heterogeneous cell populations and the degree of plasticity with which cells can transit from one state to another. The answer to this question, which will likely require assessment of longitudinal single-cell genotype/phenotype after exposure to therapeutics (both *ex vivo* and *in vivo*), will have important implications on the rational design of new drug combinations. The phenotype of cells that persist after therapy also portends a challenge and an opportunity for the field. These cells have sometimes been described as cancer stem cells and their biology has been linked to primitive stem/progenitor cells that occur in normal tissue differentiation and development. Clearly, the cells that persist after some therapies do exhibit hallmarks of undifferentiated cells. However, the application of newer interventions that can target these more primitive cells has revealed that persistent cells after therapy can also adopt a more differentiated cell state. Hence, the phenotype of cells that can persist after treatment may depend greatly on the therapeutic target and regimen used, and it has become clear that additional insights will be required to uncover the true definition of a cancer stem cell. These insights are likely to emerge as a broader diversity of therapeutics are studied in both laboratory and clinical settings.

Some of the resistance mechanisms that are displayed by persistent cells are particularly problematic because there are no currently available therapies that are effective in treating tumors with such features, so significant drug development and/or drug repurposing options are greatly needed. In addition, from this tumor complexity and pan-resistant disease features, it is clear that drug combinations are needed in nearly all cases for durable disease control. Indeed, a great deal of work has been done to predict and test effective combinations, including the development of analytical approaches to understand synergistic combinations, where the effect of the two-drug combination is superior to the effect one would predict from adding the activity of each single-agent (187–189). This presents another challenge, because the pairwise combination of all existing drugs yields a series of drug pairs that is far too large to test clinically and even in many preclinical models. Adding to this complexity, it is also clear that combinations of two drugs may not be sufficient and, in some cases, drug combinations may be optimally administered sequentially rather than in unison. Determining optimal and safe dosing, sequencing, and partnering of these multidrug cocktails will require substantial biological insights to reduce the myriad of potential scenarios, and the application of advanced computational approaches such as artificial intelligence and machine learning to understand drug resistance and predict effective drug repurposing and combinations will be critically important (190, 191). Altogether, these promises and challenges require a whole community approach of employing the most cutting-edge techniques to study broad aspects of cancer biology such that novel combination regimens can be deployed to improve patient outcomes.

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