# Design and reporting principles for experimental cancer evolution

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## ABSTRACT

Evolutionary processes are inherently stochastic, such that independently evolving populations exposed to identical conditions may not follow identical trajectories. In cancer, this stochasticity contributes to diverse resistance pathways in tumor cell populations exposed to the same therapeutic pressures. Experimental evolution provides a controlled framework for studying these dynamics by exposing cells to sustained drug treatment and allowing resistance to evolve over time. While powerful, insufficient documentation of experimental design can obscure biologically meaningful stochasticity and hinder comparisons across studies. Here, we argue for the importance of rigorous experimental design and transparent reporting in cancer experimental evolution. We describe eight key factors that should be considered when designing an experimental evolution study: (1) choosing a model system, (2) methodology for tracking evolution, (3) defining evolutionary replicates, (4) selecting treatment dose, (5) determining initial seeding density, (6) defining evolutionary cycles, (7) selecting experiments to perform at each cycle, and (8) deciding when to end the experiment. Careful consideration of these factors improves repeatability and facilitates identification of deterministic and contingent features of resistance evolution. For cancer biologists, these design choices determine whether resistance mechanisms, collateral sensitivities, persister states, and clinically actionable vulnerabilities can be interpreted as reproducible biology rather than protocol-specific artifacts.

## Introduction

Cancer is an evolutionary process[1–4]. Under therapeutic pressure, tumor populations adapt through multiple evolutionary paths that can converge on treatment resistance[5,6]. Despite this inherently dynamic process, resistance is frequently studied as a static experimental endpoint, after the phenotype of interest has fully emerged. Although endpoint analyses can characterize the resulting resistant state, they provide limited information about the selective pressures, intermediate states, and evolutionary trajectories that produced it. Understanding therapeutic failure therefore requires studying not only the resistant phenotype, but also how resistance emerges and changes over time.

Experimental evolution directly addresses this limitation by enabling cancer populations to be studied as dynamic, evolving systems (Fig. 1a–c). By maintaining populations under defined selective pressures over extended periods, researchers can repeatedly sample populations as resistance develops and connect phenotypic and molecular changes to their evolutionary

history. Experimental evolution has been applied across multiple cancer types, including Ewing sarcoma[7], non-small cell lung cancer[8–12], acute lymphoblastic leukemia[13], and breast cancer[14]. These studies have used experimental evolution to investigate clonal dynamics[14], identify predictive drug-response signatures[7,15], explore collateral sensitivity and treatment strategies[10], and characterize the evolution of cancer aneuploidy[16]. Together, this work demonstrates the utility of experimental evolution for resolving the temporal and contingent nature of cancer adaptation.

A key strength of experimental evolution is the degree of control it affords. Drug dose, treatment schedule, environmental conditions, and population size can be manipulated while populations are sampled longitudinally. This enables direct observation of resistance as it develops, supports evolutionary replication, and facilitates molecular and phenotypic characterization over time. However, this control also makes evolutionary outcomes sensitive to experimental design. Choices such as dosing strategy, passage frequency, population size, culture conditions, and sampling schedule can alter mutation supply, selection strength, and population dynamics, thereby influencing the evolutionary trajectories that are ultimately observed.

Despite its growing use, experimental evolution is not a single, standardized approach. Some design decisions are straightforward, whereas others are more subtle and vary widely across studies; both, however, are often inconsistently reported or insufficiently justified. More broadly, recent work in ecology and evolution has emphasized the importance of explicitly aligning experimental design with the scale of biological inference and the evolutionary questions being addressed[17,18]. While designing our own cancer experimental evolution systems, we identified recurring decisions that can substantially shape evolutionary outcomes but are rarely discussed in detail. To examine how these choices are implemented in practice, we systematically surveyed experimental evolution studies across the cancer literature.

Two studies provide particularly illustrative examples of contrasting experimental strategies (Fig. 1d,e; Supplementary Note 1). Zhao et al.[13] used serial dose escalation and repeated collateral sensitivity profiling, whereas Acar et al.[8] maintained large populations without routine passaging to preserve clonal diversity during treatment. Neither represents a universal best practice; rather, together they demonstrate how experimental design can be aligned with distinct scientific questions. More generally, parallel evolutionary replicates derived from a common ancestor can experience the same selective environment and converge on a resistant phenotype while traversing distinct trajectories across a fitness landscape (Fig. 1a–c). Experimental evolution therefore provides a framework for studying not only whether resistance emerges, but also the diversity and repeatability of the evolutionary paths leading to it.

Variation in experimental design extends across the broader literature. Across 60 long-term cancer evolution studies that we systematically examined, designs differed in model system, treatment strategy, dose escalation, passaging, barcoding, and molecular profiling (Fig. 2; Supplementary Tables 1 and 2). Multiple correspondence analysis showed that studies broadly grouped according to their scientific objectives, suggesting that methodological variation partly reflects the questions investigators seek to answer. These choices are therefore not simply technical details: they determine which evolutionary processes can be observed and how the resulting data can be interpreted.

At the same time, important methodological details are often distributed across the main text, figures, and supplementary

materials or are incompletely reported, complicating comparisons among studies. This is particularly consequential in evolution experiments because independently evolving populations are not expected to follow identical trajectories. Without sufficient information about experimental conditions, biologically meaningful stochasticity can be difficult to distinguish from protocol-dependent variation.

Here, we synthesize lessons from the cancer experimental evolution literature into eight interconnected considerations for designing and reporting an evolution experiment (Fig. 3): (1) choosing a model system, (2) tracking evolution, (3) defining evolutionary replicates, (4) choosing a selection dose, (5) determining initial seeding density, (6) defining evolutionary cycles, (7) selecting repeated measurements, and (8) defining the experimental endpoint. We propose the term "evolutionary replicate" to distinguish independently evolving populations from other forms of replication; our definition is provided in Box 1. These considerations are not intended as prescriptive rules; rather, they provide a framework for aligning experimental design with the biological question and for reporting the information necessary to interpret, compare, and reproduce cancer evolution studies.

**Defining evolutionary replicates**

In an evolution experiment, clearly distinguishing among types of replication is important for interpreting evolutionary dynamics across populations. We use the term "**evolutionary replicate**" to describe a biological replicate undergoing evolution independently in parallel from the same ancestral clone or starting population. Although evolutionary replicates experience the same intended experimental conditions, they may follow distinct adaptive trajectories over time. Importantly, they must remain independent throughout the experiment; pooling populations during passaging can introduce pseudoreplication by allowing stochastic events arising in one lineage to propagate across otherwise independent populations[19]. Evolutionary replication is distinct from biological and technical replication. **Biological replicates** represent biologically distinct samples and capture underlying biological variability, whereas **technical replicates** are repeated measurements of the same sample used to estimate variability introduced by experimental procedures or instrumentation[20]. Although technical replication can reduce measurement uncertainty, increasing the number of independent biological or evolutionary replicates is generally more informative for characterizing the repeatability, contingency, and diversity of resistance evolution.

## Design variation in cancer experimental evolution

Three features of the literature particularly illustrate why experimental design requires careful consideration: selection dose, population bottlenecks and experimental endpoints.

### *Selection pressure varies substantially among studies*

Published cancer evolution studies use diverse dosing strategies, ranging from fixed concentrations maintained for months[15,21,22] to progressive dose escalation from fractions of the ancestral $IC_{50}$, the concentration that reduces cell viability by 50%, to substantially higher concentrations[9–11,13,23–27]. Evidence across experimental systems indicates that selection intensity can affect both the rate and nature of adaptation[28–32]. In cancer models specifically, increasing drug exposure can produce sequential intermediate states along a resistance continuum[30], while alternative treatment schemes can select distinct resistance mechanisms even against the same therapy[31]. Thus, dose should be considered an evolutionary parameter rather than merely a technical feature of drug treatment.

#### *Population size and passaging alter evolutionary opportunity*

Passaging regimen and effective population size are less consistently reported but are similarly important. Serial passage creates recurrent population bottlenecks whose size and frequency influence genetic drift, mutation retention and the probability that beneficial variants survive. Work in microbial experimental evolution has demonstrated that bottleneck severity can alter the rate and trajectory of adaptation, although these effects interact with selection strength and other environmental conditions[33–38]. The same evolutionary principles are relevant to cancer populations, in which population size determines mutation supply and influences competition among subclones. Reporting the number of cells transferred, dilution ratio, culture vessel and related parameters is therefore necessary for interpreting evolutionary trajectories.

#### *Experimental endpoints determine which states are observed*

Studies also differ substantially in when evolution is considered complete. Published endpoints include fixed durations or passage numbers, failure of cellular outgrowth, achievement of a specified drug concentration, predefined fold increases in resistance and completion of clinically motivated treatment cycles[13,15,39–43]. These choices influence which stages of adaptation are captured. Early termination may emphasize transient tolerance or partial resistance, whereas prolonged selection can permit secondary mutations, compensatory adaptations and stabilization of resistant states. A meaningful endpoint should therefore be defined relative to the scientific question, using criteria such as a phenotypic threshold, stability of resistance, duration of selection, or a combination thereof. The endpoint criteria should be explicitly reported.

Together, the heterogeneity of dosing, population management and endpoints reinforces a central principle: there is no universally optimal cancer evolution protocol. Instead, experimental choices should be intentional, internally consistent and sufficiently documented to establish what evolutionary processes the experiment was capable of observing.

## Considerations for designing a cancer evolution experiment

We propose eight considerations that collectively connect the scientific question to the evolutionary processes an experiment can resolve **(Figure 3)**. Because these decisions interact, they should ideally be considered together during experimental planning rather than treated as independent methodological choices.

### 1. Choosing a model system

The research question should determine the appropriate model system and experimental design[44,45]. Cancer models differ not only in biological complexity but also in the evolutionary processes that can feasibly be observed. Established cell lines provide experimental control, scalability, and compatibility with high replication, repeated perturbation, and longitudinal sampling. More complex systems, such as co-culture, organoids, patient-derived models, and *in vivo* models can capture selective pressures arising from stromal or immune interactions, spatial heterogeneity, metastatic progression, and pharmacokinetic variation, but often at the expense of throughput and experimental tractability.

The most biologically realistic system is therefore not necessarily the most informative for every evolutionary question. Large-scale cell culture may be advantageous for studying mutation supply, repeatability, and clonal competition, whereas

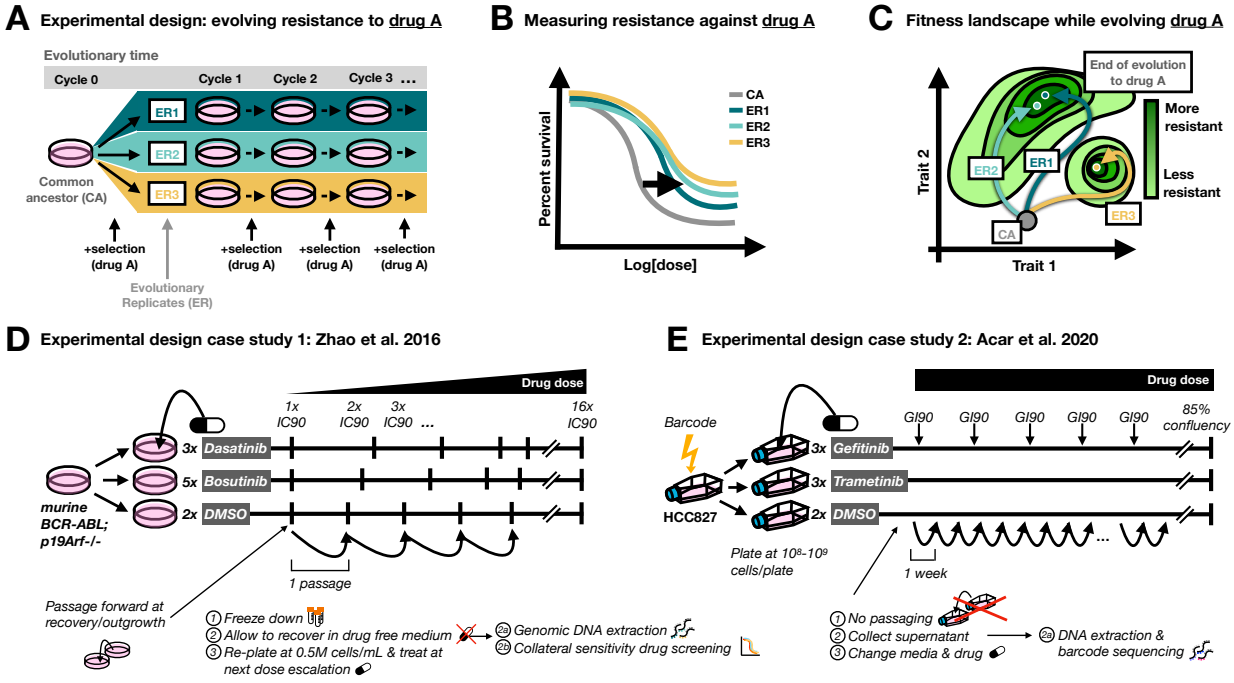


**Figure 1. Conceptual and experimental frameworks for studying cancer evolution.**. **(A)** Schematic of three evolutionary replicates (ER1—ER3) derived from a common ancestor and independently exposed to drug A. **(B)** Longitudinal tracking of resistance, illustrated by increasing $IC_{50}$ to drug A across treatment cycles. **(C)** Evolutionary replicates traversing a fitness landscape under drug selection. Despite experiencing the same selective pressure and converging on resistance, replicate populations may follow distinct evolutionary trajectories. **(D)** Experimental design of Zhao et al.[13], in which ten replicate BCR–ABL; p19Arf$^{-/-}$ populations underwent escalating drug exposure until reaching 16×$IC_{90}$ or failure to regrow, with longitudinal cryopreservation, drug screening and DNA collection. **(E)** Experimental design of Acar et al.[8], in which eight HCC827 populations were maintained under a fixed $GI_{90}$ exposure, or the concentration of a compound that induces 90% growth inhibition, until the flask reached 85% confluence, with DNA collected longitudinally from culture supernatant.

questions centered on microenvironmental selection may require more complex models. Compatibility with longitudinal approaches such as repeated passaging, barcoding, lineage tracing, and frequent sampling should also be considered. Prolonged culture itself can alter transcriptional or phenotypic states[46–51]; for example, the high transcriptional plasticity of Ewing sarcoma may favor live-cell approaches when preservation of transcriptional state is important[49–51]. Model selection should therefore balance biological relevance, experimental tractability, and the evolutionary variables the study aims to preserve.

## 2. Tracking evolution

The defining advantage of experimental evolution is the ability to observe change through time, rather than infer evolutionary history solely from an endpoint. The method used to track evolution should match whether the question focuses on population-level phenotypes, clonal dynamics, or both.

Population-level tracking can be performed using cell-tracking and imaging approaches that monitor changes in cell number, growth, morphology, and spatial organization over time[52,53]. Fluorescent labeling can facilitate automated cell counting and reveal population growth patterns or clonal expansions[54,55], while fluorescent reporters can track functional changes, including

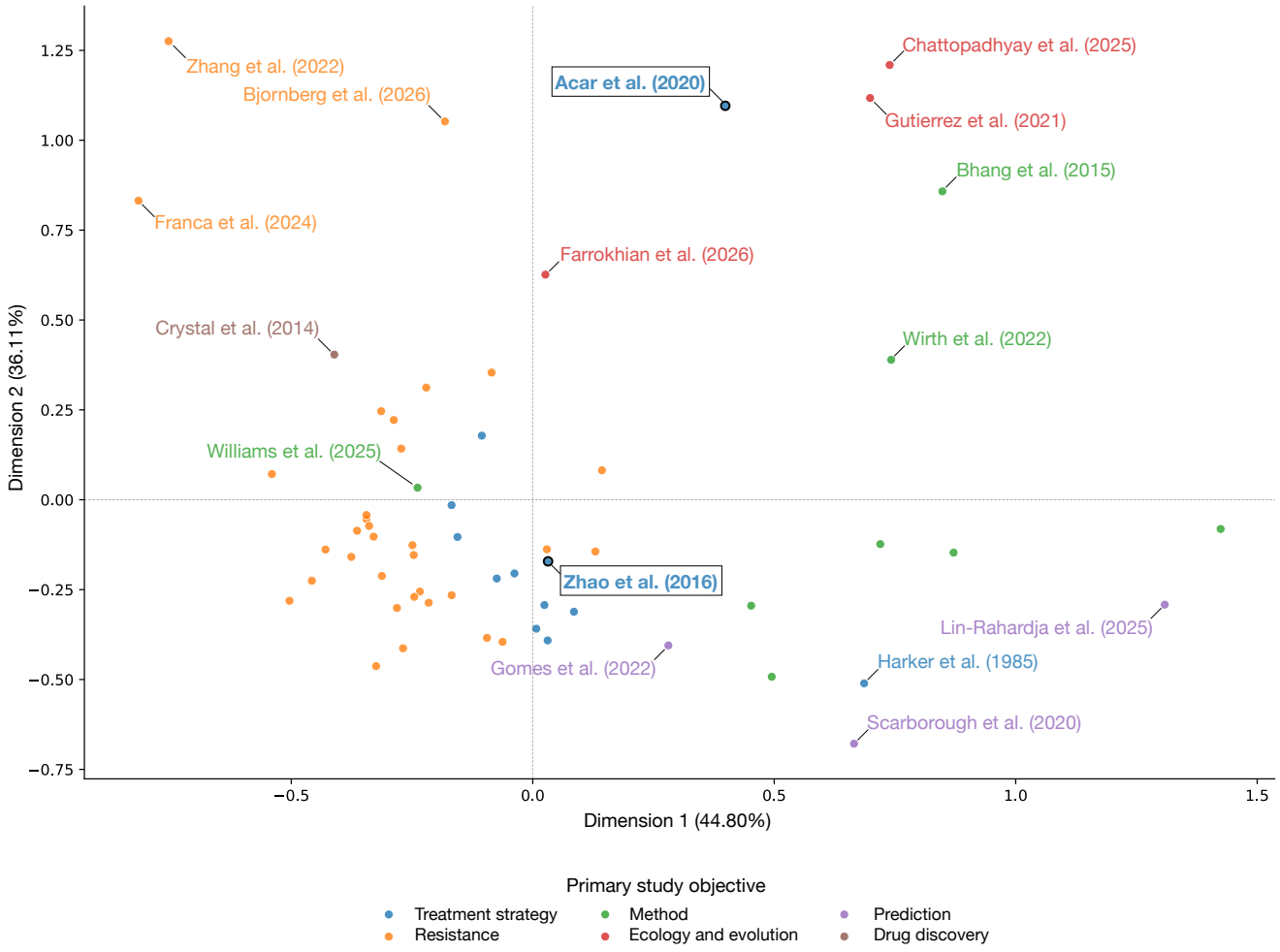


**Figure 2. Multiple Correspondence Analysis of experimental evolution study design.** Multiple Correspondence Analysis (MCA) was used as an exploratory approach to visualize similarities and differences across 60 experimental evolution studies conducted over extended timescales (on the order of months). Each point represents a single study, with distances reflecting similarity in experimental design: studies positioned closer together share more methodological features, whereas those further apart are more dissimilar. Points are color-coded by primary study objective (see legend). Studies in this analysis cluster roughly by their primary study objective. Papers that appeared to diverge from their respective cluster were identified and labeled. The MCA incorporated categorical variables capturing key experimental parameters, including treatment type, study objective, cancer type, barcoding, drug dosing strategy, dose escalation, passaging regimen, and molecular readouts (i.e., DNA- and RNA-based sequencing approaches). MCA was performed on the corresponding indicator matrix using singular value decomposition. Axis values represent the proportion of total inertia (variance) explained following Benzecri correction. The full dataset and encoding scheme are provided in Supplementary Tables 1 and 2. Analyses were conducted in Python using the prince MCA package.

transitions toward stem-like states[56,57]. Repeated drug-sensitivity assays can establish when resistance emerges and whether it develops gradually or abruptly, while longitudinal transcriptomic profiling can reveal the timing of molecular changes accompanying adaptation[58,59]. Cell-cycle reporters such as FUCCI provide an additional means of monitoring transitions between proliferative and quiescent states during treatment[60,61]. Together, these approaches capture dynamic population-level and phenotypic changes that may be missed by endpoint measurements alone.

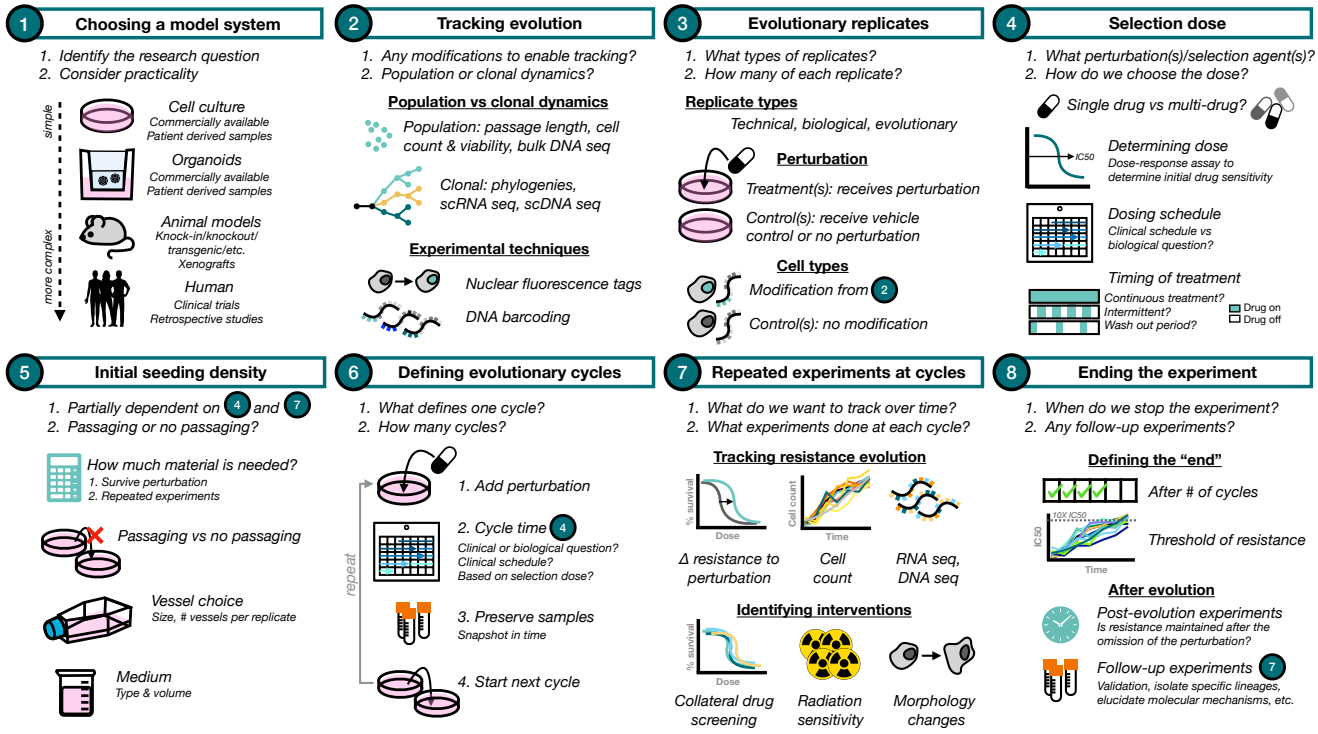


**Figure 3. Eight considerations for designing a cancer evolution experiment**. **(1)** Choosing a model system involves identifying the research question and considering practicality. **(2)** Tracking evolution is important to study the model system over time. Two ways to track are population dynamics and clonal dynamics. **(3)** Defining evolutionary replicates are key. This involves deciding what type of replicates are necessary and how many of each. **(4)** Choosing a selection dose for the perturbation may take into account many things such as the clinical schema, dosing time and level, and treatment strategies. **(5)** Deciding the initial seeding density of the experiment is key for further down assays and to keep the experiment going. **(6)** Defining evolutionary cycles include understanding when to add selection/perturbation to the model system and what assays will be done on a specific time interval. **(7)** What experiments will be done at every cycle to track the evolution of resistance or evolution of oncogenesis? **(8)** Ending the experiment can be at a selected time interval, cycle interval, or after the model has reached a threshold of resistance. After the evolution experiment, post-treatment experiments or follow-up experiments using the temporally preserved samples generated can be done as well.

Population averages, however, can obscure heterogeneous evolutionary trajectories. Rare subclones can expand under treatment, while interactions among subpopulations may influence resistance[62,63]. Cellular barcoding and DNA-based lineage tracing enable individual lineages to be tracked throughout treatment[64–69]. Extensions combine lineage tracing with single-cell molecular profiling or CRISPR-based recording to resolve cellular histories at higher resolution[70–72]. Complementary approaches quantify lineage dynamics in vivo and investigate tumor initiation, including Tuba-seq and genetic lineage-tracing models[73–78].

### *Tracking clonal dynamics*

Tumor heterogeneity drives cancer evolution, as rare subclones may harbor traits that enable tumorigenesis or therapy resistance. Clonal competition and cooperation further shape evolutionary outcomes[63,79]. Understanding these dynamics requires examining individual clones rather than population averages alone.

Cellular barcoding uses heritable nucleic acid identifiers to track thousands of clones across time, space, and treatment

conditions[64–66]. These approaches have been applied to tumor heterogeneity, drug resistance, immunotherapy response, metastasis, and progression[67–70,74]. CRISPR-based lineage tracing introduces genetic scars to reconstruct cell division histories and, when combined with scRNA-seq, link lineage histories to transcriptional states[71,72]. Tuba-seq extends clonal quantification *in vivo* by combining tumor barcoding with ultra-deep sequencing to measure clonal fitness and tumor size distributions[73]. Although these approaches have primarily been used to study tumorigenesis and tumor initiation[75–77], some studies have begun applying them to treatment response in vivo. For example, Foggetti et al. used Tuba-seq to identify genotype-specific responses to osimertinib in a mouse model of EGFR-driven, Trp53-deficient lung adenocarcinoma[78]. Extending these approaches to prolonged therapeutic exposure could enable the study of clonal dynamics during resistance evolution in vivo.

## 3. Evolutionary replicates

Replication in an evolution experiment must capture the type of variation relevant to the scientific question. Biological replicates represent distinct biological samples, technical replicates estimate assay variability, and evolutionary replicates are populations evolving independently from a common starting population[20]. The latter are particularly important because stochastic divergence among populations is itself an outcome of interest.

Controls should similarly reflect the sources of variation introduced by the experimental design. Vehicle controls can distinguish drug effects from solvent effects, while labeling or transduction controls can account for perturbations introduced by fluorescent tags or barcodes[80,81]. Unmodified or naïve populations can provide an additional baseline where appropriate. Importantly, control populations maintained for the duration of the experiment can also reveal changes caused by prolonged culture rather than the selective treatment itself.

The number of evolutionary replicates should be determined by the inference the study aims to make. Designs focused on an average treatment effect have different requirements from studies intended to characterize the distribution or repeatability of evolutionary outcomes. Large numbers of independent populations can reveal heterogeneous trajectories that would be invisible with only one or two lines, although the scale achievable in microbial experimental evolution[82] is often impractical in cancer models. Replicate number should therefore balance feasibility with the desired resolution of evolutionary variability, while anticipating statistical approaches appropriate for longitudinal and dose-response data[83–88].

## 4. Choosing a selection dose

Selection dose encompasses both drug concentration and the temporal pattern of exposure. Together, these parameters determine the strength and structure of selection acting on the evolving population.

Drug concentration is commonly informed by an initial dose-response assay and expressed relative to the ancestral sensitivity of the model. However, an $IC_{50}$ is not intrinsically the optimal evolutionary dose, and maximum tolerated doses have also been proposed as a selection strategy[89]. Selection must be strong enough to favor adaptation while retaining a sufficiently large surviving population for evolution to proceed. Experimental evidence suggests that selection strength can influence both the stability and genetic basis of resistance[90]. Conversely, clinically motivated studies may prioritize exposures approximating

patient plasma or tissue concentrations rather than a particular *in vitro* response threshold[91]. These objectives are not equivalent, and the rationale for choosing one should be explicit.

The temporal schedule is equally important. Continuous treatment maintains sustained selection, whereas intermittent treatment introduces periods of recovery that may alter competition among sensitive, tolerant, and resistant populations. Clinical regimens can motivate these schedules, particularly for cyclic chemotherapy or multidrug treatment, whereas simplified exposures may be preferable when the goal is to isolate the evolutionary consequences of a single agent.

Dose escalation offers another strategy, progressively increasing selection as adaptation occurs. Fixed and escalating designs answer somewhat different questions: a fixed environment allows adaptation to a defined selective pressure to be studied, whereas escalation continually changes that environment and can select populations capable of tolerating increasingly strong treatment. Because selection strength can alter both evolutionary tempo and mechanism, studies should report not only the final maintenance concentration but also the initial dose, escalation criteria, the timing of changes, exposure duration and drug-free intervals.

## 5. Choosing an initial seeding density

Population size determines both experimental feasibility and evolutionary opportunity. Sufficient cells must survive treatment, sustain the evolving population and provide material for planned measurements, but the number of cells also affects mutation supply and the diversity available to selection.

Serial passaging introduces repeated bottlenecks, as only a subset of cells is transferred between cycles. Bottleneck severity depends on the number of cells transferred and can determine whether rare beneficial variants are retained. Additional bottlenecks and selective pressures may arise during model establishment, genetic modification, antibiotic selection, and freezing and thawing. These procedures can shape genomic evolution and, consequently, the resulting phenotypes[92–94]. They should therefore be considered part of the model's evolutionary history rather than purely logistical manipulations.

Some experimental systems can minimize passaging. For example, Acar et al.[8] used large HYPERflask cultures to maintain populations without routine replating, thereby preserving large population sizes during treatment. Such designs are not feasible for every study, and passaging itself is not inherently problematic; rather, its evolutionary consequences should be recognized and reported.

Population requirements also interact with practical considerations. Transcriptomic profiling, lineage tracking and collateral drug screens may require substantial material and therefore influence vessel size or the number of parallel cultures. Conversely, a trade off of high-replicate experiments may favor smaller vessels, due to lack of incubator space or other practical considerations. Initial seeding density, number of cells transferred, vessel type, medium volume and related culture parameters should be standardized across replicates and reported sufficiently to reconstruct the population bottlenecks imposed during the experiment.

## 6. Defining evolutionary cycles

Long-term evolution experiments involve repeated treatment, growth, sampling and measurement, but the temporal unit represented by a "cycle" varies among studies. We define an evolutionary cycle as the interval between successive treatment or measurement points used to structure longitudinal evolution. Explicitly defining this unit provides a common reference for comparing populations through time.

In practice, a cycle may be anchored to treatment application, media replacement, passage or a biological milestone. Fixed cycles use a predetermined interval and may be appropriate when reproducing a clinical regimen or coordinating scheduled measurements. Dynamic cycles instead depend on the evolving population reaching a criterion such as cell density or confluency. The latter allows slower- and faster-growing populations to progress according to their biological state, although elapsed chronological time may then differ among replicates.

Neither strategy is universally preferable. What matters is that the event defining the beginning and end of a cycle, the timing of treatment, and the criteria for progression are specified. Reporting both cycle number and elapsed time can be particularly informative when populations diverge in growth rate. Cycles also provide natural opportunities for sample preservation. Cryopreserved populations or collected molecular material create a longitudinal archive that can support retrospective analysis, validation and application of assays developed after the experiment began. Preservation itself can introduce bottlenecks or alter cellular phenotypes[47,48], so investigators should distinguish measurements made directly from evolving cultures from those performed after freeze–thaw recovery.

## 7. Repeated measurements during the experiment

Longitudinal measurement transforms a resistant endpoint into an evolutionary trajectory. Assays conducted at multiple cycles can reveal when resistance emerges, whether it develops monotonically or fluctuates, and when molecular or phenotypic changes precede stable resistance.

Measurements can broadly serve two purposes. The first is to characterize the evolving system itself; for example, cell number, growth rate, morphology or sensitivity to the selection agent. The second is to identify changes that accompany evolution and may create new therapeutic opportunities, including collateral drug responses, radiation sensitivity, transcriptomic states and genomic alterations. Repeated measurements of both types can connect the timing of resistance with mechanisms and vulnerabilities that emerge during adaptation.

The choice of assay should account for temporal resolution, throughput and whether material must be analyzed live. Dose-response assays against the selection agent can confirm that the intended phenotype is evolving and can inform dose escalation. Molecular sampling can capture transient states that may disappear by the endpoint. Where cryopreservation alters the phenotype of interest, collection or measurement during active evolution may be necessary.

Assay resolution should likewise match the scientific question. High-resolution dose-response screens can improve quantitative precision but reduce the number of drugs, populations or time points that can feasibly be tested. Automated imaging, liquid handling and plate-based measurements can increase throughput, but technological capability should not

dictate experimental design independently of the biological question. The goal is to obtain sufficient temporal and phenotypic resolution to distinguish meaningful evolutionary change while maintaining a sustainable experiment.

### 8. Ending the experiment

An evolution experiment requires an explicit definition of its endpoint. Depending on the question, this may be a fixed duration or number of cycles, achievement of a predefined resistance threshold, sustained growth at a specified drug concentration, or another biologically motivated criterion. Because endpoint choice determines which stages of adaptation are captured, it should ideally be established before the experiment while allowing predefined contingency plans if evolution proceeds unexpectedly quickly or slowly.

The endpoint of selection need not be the endpoint of observation. Drug withdrawal after evolution can test whether resistance is stable or reversible and can reveal fitness costs or reversion toward ancestral sensitivity. Follow-up measurements may include growth, drug sensitivity, morphology or competition between evolved and ancestral populations.

Longitudinal sample preservation greatly expands the analyses available after the experiment. Archived populations and molecular samples can be used to identify resistant precursor states, reconstruct evolutionary trajectories or validate candidate mechanisms. Lineage-tracing approaches such as ClonMapper similarly enable retrospective investigation of clonal dynamics during treatment[95,96].

The experimental endpoint should therefore be viewed as part of the study's biological definition of resistance rather than simply the date on which culture ends.

## Discussion

Experimental evolution is not a single protocol but a framework of interconnected design choices that shape mutation supply, selection, and population dynamics. Given the influence of these decisions on experimental outcomes, thorough documentation and reporting are essential for ensuring reproducibility and comparison. Similarly, careful consideration of experimental parameters is critical when designing an experimental evolution study, as the choices made directly influence both the evolutionary trajectories observed and the biological questions that can be meaningfully addressed.

Because evolution is inherently stochastic, replication and documentation are required to distinguish meaningful biological contingency from protocol-driven variation. Even under nominally identical conditions, independently evolving populations are not expected to follow identical evolutionary paths. This variability is not a flaw of the approach, but rather a defining feature of evolutionary processes. However, insufficient documentation of experimental design makes it difficult to distinguish biologically meaningful stochasticity from variation introduced by unreported or uncontrolled experimental parameters. Conducting more well-documented studies, particularly those with multiple evolutionary replicates, will be essential for identifying robust evolutionary patterns, assessing repeatability, and disentangling contingent versus predictable features of resistance evolution.

Experimental evolution occupies a distinct position among cancer modeling approaches because it specifically enables the study of evolutionary dynamics over time. Approaches centered on single perturbations, such as knockout or overexpression

studies, are powerful for identifying causal mechanisms independent of the evolutionary processes through which they emerge. In contrast, clinical and patient-derived systems capture the complexity of tumor evolution, including microenvironmental interactions, immune dynamics, and heterogeneous selective pressures but can be difficult to interrogate longitudinally or mechanistically. Experimental evolution instead addresses a complementary question: how does resistance emerge under sustained selective pressure over time? By exposing populations to defined treatments across repeated cycles of growth and selection, these approaches make it possible to study the dynamics, repeatability, and diversity of adaptive trajectories leading to resistance. Importantly, the resistance observed in these systems is not engineered through predefined genetic perturbations beyond the treatment itself but instead emerges through an open-ended evolutionary search process that may uncover multiple competing or unexpected adaptive solutions. As a result, these systems can uncover adaptive solutions that may not have been predicted *a priori* from existing biological knowledge.

A related challenge is how resistance itself is defined, which can vary widely across studies, experimental systems, and clinical contexts. Clinically, resistance is often measured as disease progression or treatment failure, but the underlying biology can differ depending on the therapy. For cytotoxic chemotherapies, resistance usually reflects broad survival adaptations, such as increased stress tolerance, enhanced DNA repair, or drug efflux[97]. In these cases, laboratory studies often define resistance using measurable phenotypes like shifts in dose–response metrics ($IC_{50}$, $EC_{50}$), the ability to proliferate at a fixed drug concentration, or survival after prolonged drug exposure. For targeted therapies, resistance is often driven by specific molecular changes, such as mutations in the drug target, pathway reactivation, or bypass signaling, which can be tracked as molecular markers in addition to phenotypic assays. These definitions emphasize different aspects of resistance and are not directly interchangeable. Clearly stating how resistance is defined and why that definition is appropriate for a given model system and therapeutic context is critical for interpreting evolutionary dynamics and comparing results across studies.

Beyond the endpoint-based definitions of resistance, resistance evolution is increasingly recognized as a process that can involve substantial phenotypic plasticity in addition to stable genetic change. Drug-tolerant persister states, epigenetic remodeling[98,99], and stem-like programs[100–102] can enable populations to survive therapy in the absence of fixed resistance mutations, particularly during early stages of selection. Importantly, resistance evolution also unfolds within a dynamic ecological context, where interactions among coexisting subpopulations can influence the emergence, persistence, and expansion of both genetic and non-genetic resistance states[103]. Although these transient or reversible states may strongly shape subsequent evolutionary trajectories, they are often difficult to detect using bulk population assays alone. Capturing such dynamics therefore requires experimental designs and measurement strategies that are sensitive to non-genetic heterogeneity and temporal changes in cell state. While the design considerations we describe here primarily emphasize genetic mechanisms of resistance (such as the use of clonal populations, bulk measurements of population-level responses, and endpoints defined by shifts in drug sensitivity), additional work is needed to systematically incorporate plastic and epigenetic resistance states and to determine their stability over evolutionary time. Approaches such as evolving barcode systems, single-cell transcriptomic and epigenomic profiling, longitudinal multi-omics, and fluorescent lineage tracing of defined cancer cell subpopulations[104]

are beginning to reveal how resistance-associated states emerge, expand, and, in some cases, stabilize. In parallel, clinical technologies leveraging similar evolutionary principles are being developed to track resistance dynamics in patients. One example is CloneSeqSV, which combines single-cell whole-genome sequencing with targeted deep sequencing of clone-specific structural variants in time-series cell-free DNA to follow resistance evolution *in vivo*[105]. Using this approach, the authors followed 18 patients with high-grade serous ovarian cancer from diagnosis through recurrence and showed that resistance most often arose through the selective expansion of a single or small subset of clones present at diagnosis. The integration of lineage plasticity and epigenetic techniques into experimental evolution research holds significant promise for advancing our understanding of treatment resistance. Because these approaches are relatively new within the experimental evolution field, rigorous documentation will be essential for establishing best practices for studying lineage plasticity and epigenetic dynamics in this context. At the same time, incorporating these methodologies increases experimental complexity, underscoring the need for a standardized framework for reporting experimental design.

In this paper, we have focused on experimental evolution as it pertains to treatment resistance; however, many of the same experimental parameters also apply to the study of oncogenesis. Previous work has shown that careful experimental control can reveal both deterministic and contingent features of cellular transformation[106], particularly in longitudinal systems using human epithelial- or fibroblast-derived models. The logic underlying experimental perturbations in oncogenesis closely parallels that of resistance evolution studies: interventions must be strong enough to drive meaningful change but not so extreme that they eliminate the population's capacity to adapt. For example, some considerations in experiments modeling oncogenesis may include the following: a cycle could be defined more broadly as a fixed interval such as a set number of days during which repeated measurements are taken to monitor the gradual acquisition of oncogenic traits. This approach allows researchers to capture stepwise changes in cellular behavior, morphology, or gene expression, and to link specific perturbations to observed transitions toward a malignant phenotype. Defining cycles in this way ensures that longitudinal observations are comparable across experiments and provides a structured framework for analyzing the dynamics of cellular evolution. Time-course experiments, including long-term organoid cultures that capture multi-phase developmental trajectories[107], illustrate how stepwise accumulation of alterations shapes malignant transformation over time. Experimental evolution and longitudinal modeling provide powerful approaches for dissecting the contributions of individual oncogenic events, while also highlighting the broader principle that careful and consistent experimental design is essential for understanding tumor evolution across all stages of disease.

More broadly, experimental evolution enables us to ask questions about whether resistance trajectories can be anticipated, delayed, or even steered through time-varying treatment strategies. For example, recent work has explored optimizing drug holidays to constrain population growth[103], modeling resistance under fluctuating drug environments[108], and using theoretical and computational approaches to design treatment strategies that guide or optimize evolutionary trajectories[22,109,110]. These approaches highlight the potential to move beyond describing resistance toward actively shaping its evolutionary dynamics.

By explicitly modeling resistance as a dynamic and historically contingent process, experimental evolution studies can help

identify early markers of resistance, characterize alternative evolutionary routes to treatment failure, and reveal tradeoffs or vulnerabilities that emerge during adaptation. Understanding how resistance trajectories depend on treatment schedules, dosing strategies, and population bottlenecks provides a conceptual framework for anticipating failure rather than reacting to it. While experimental evolution cannot recapitulate the full complexity of tumors in patients, it can generate testable hypotheses about resistance dynamics, collateral sensitivities, and adaptive treatment strategies that inform preclinical studies and clinical trial design. In this way, experimental evolution serves not as a direct surrogate for patient outcomes, but as a discovery engine for evolutionary principles that may guide more rational and evolution-informed therapeutic strategies.

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## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

The datasets generated and analyzed during the current study are included in the supplementary materials. The manually curated literature codebook and dataset used for multiple correspondence analysis are provided in Supplementary Tables 1 & 2.

### Competing interests

The authors declare no competing interests.


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### Authors' contributions

M.N.D. led study conceptualization, manuscript drafting, and preparation of Figures 1 and 3. M.N.D. and J.I. prepared Figure 2 and incorporated revisions. M.N.D., J.I., K.L.R., and J.W. prepared Supplementary Tables 1 and 2. J.A.A. and M.A.R.S. contributed to study conceptualization, manuscript writing, and revisions throughout manuscript preparation. J.G.S., A.M., M.A.R.S., J.A.A., and K.L.R. provided editorial feedback. J.G.S. and M.A.R.S. supervised the project. All authors reviewed and approved the manuscript.


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## Supplementary Materials

### Supplementary Note 1

Zhao et al. (2016)[13] used a fusion-positive, murine-derived acute lymphoblastic leukemia cell line containing the *BCR-ABL1* fusion to investigate adaptive responses to targeted therapy **(Figure 1d)**. The study focused on two drugs, dasatinib and bosutinib, both second-generation tyrosine kinase inhibitors targeting Bcr-Abl and Src kinases, with DMSO-treated cells as the vehicle control. A total of ten evolutionary replicates were maintained in parallel: five treated with dasatinib, three with bosutinib, and two with DMSO. Cells were plated at 0.5 million cells/mL. If the goal is to generate a resistance profile, as it was in this study, dose escalation is logical because it progressively challenges the cells with higher drug concentrations. They initially treated at $1\times IC_{90}$. Upon recovery and outgrowth (defined as reaching 4–5 million cells/mL) subsets of the cultures were either (1) frozen, (2) allowed to recover in drug-free medium, or (3) re-plated at 0.5 million cells/mL and treated at the next dose escalation ($2\times IC_{90}$, $3\times IC_{90}$, and so on, up to $16\times IC_{90}$). From step (2) "allowed to recover in drug-free medium," the authors performed pharmacological screening against a panel of drugs and extracted genomic DNA for PCR amplification and Sanger sequencing of the ABL1 kinase domain to monitor resistance-associated mutations. These repeated measurements throughout time allowed the researchers to examine the system's collateral sensitivity — an evolutionary tradeoff, where resistance to one drug increases susceptibility to another agent. Once cultures reached $16\times IC_{90}$ (or failed to show outgrowth), the stability of recovered cell lines was confirmed through periodic Sanger sequencing and dose–response assays for up to two months. The experiment concluded when cultures reached the maximum drug concentration or failed to grow; however, the time required to reach these endpoints was not recorded.

Acar et al. (2020)[8] used the EGFR-mutant non-small cell lung cancer line HCC827 (exon 19 deletion) to study evolutionary trade-offs and strategies to delay resistance by steering clonal trajectories **(Figure 1e)**. The authors first highlighted the limitations of of standard experimental evolution approaches involving re-plating. Through stochastic modeling, they found that small population sizes and passaging bottlenecks can strongly influence evolutionary dynamics. These effects increase genetic drift, reduce intratumoral heterogeneity, and lead to highly variable resistance trajectories across replicates. For these reasons, they adopted a no-passaging approach, combining single-cell barcoding (ClonTracer) with culture in Corning High Yield Performance Flasks (HYPERflasks) to maintain large continuous populations over time. The study investigated two drugs, gefitinib and trametinib, with DMSO-treated cells as the vehicle control. In total, eight evolutionary replicates were maintained in parallel: three treated with gefitinib, three with trametinib, and two with DMSO. Cells were plated at large populations of $10^8$–$10^9$ cells and treated at the $GI_{90}$, the dose that achieved a growth inhibition of 90%, of drug (gefitinib: 40 nM; trametinib: 100 nM). Media and drugs were refreshed weekly, and supernatant cells collected at each change were used for DNA extraction and barcode sequencing to quantify clonal dynamics over time. The experiment concluded when cultures recovered to approximately 85% confluency (DMSO: 6 days; gefitinib: 4 weeks; trametinib: 9 weeks). The authors also noted that the drug concentrations used may not always be clinically achievable in patients. By combining large population sizes with longitudinal barcode tracking, the authors were able to study how therapeutic pressure shapes clonal trajectories during

resistance evolution.

**Table 1. Codebook of dimensions used for multiple correspondence analysis of experimental evolution studies.** Definitions and categorical groupings used to annotate studies included in the literature review–based multiple correspondence analysis (MCA). Variables capture key experimental design features, including treatment modality, study objective, model system, dosing strategy, evolutionary protocol characteristics, and molecular profiling approaches. These dimensions were used to compare methodological similarities and differences across experimental evolution studies in cancer.

| Dimension | Definition | Options |
|---|---|---|
| **Type of treatment** | Primary intervention or study category involving therapeutic or non-therapeutic exposure conditions. | Targeted therapy; Chemotherapy; Radiation; Nutrients; Review; Method; Patient treatment |
| **Objective** | Primary scientific goal of the study, grouped into higher-level functional categories. | **Resistance:** Resistance mechanisms; Drug tolerance<br>**Treatment Strategy:** Collateral response; Treatment strategy<br>**Prediction:** Predictive biomarker; Gene signature<br>**Eco/Evo:** Ecology in cancer; Evolution in cancer<br>**Drug discovery**<br>**Method** |
| **Type of system** | Experimental or clinical model used to study cancer evolution. | Commercial cell line; Patient-derived cell line; Organoid; Animal; Human/clinical; Patient biopsy; N/A (method/review) |
| **Cancer type** | Broad classification of disease based on tissue or organ of origin. | Lung cancer; Leukemia; Colon cancer; Sarcoma; Ovarian cancer; Breast cancer; Cholangiocarcinoma; Prostate cancer; Liver cancer; Melanoma; Pancreatic cancer; Head and neck cancer; Neuroblastoma |
| **Barcoding** | Use of lineage tracing or clonal barcoding methods. | Yes; No |
| **Drug dosing strategy** | Framework for how drug concentration was selected or applied. | Clinical regimen (standard of care based); Evolutionary regimen (selection pressure-based); Patient sample - clinical regimen; N/A (method/review) |
| **Drug escalation** | Whether drug concentration changed during selection. | Escalate dose; Static dose |
| **Passaging** | Whether cells were periodically subcultured during experiments. | Yes; No |
| **DNA analysis** | Whether genomic assays were performed. | Yes; No |
| **RNA analysis** | Whether transcriptomic assays were performed. | Yes; No |

**Table 2. Annotated experimental design features of studies included in the multiple correspondence analysis.** Literature-curated classifications assigned to each experimental evolution study used in the MCA. Studies were manually annotated across multiple methodological dimensions to enable comparison of experimental design strategies across the cancer evolution literature.

| Paper | Treatment | Objective | System | Cancer type | Barcoding | Drug dosing strategy | Drug escalation | Passaging | DNA | RNA |
|---|---|---|---|---|---|---|---|---|---|---|
| Dhawan et al. 2017[10] | Targeted | Collateral response, treatment strategy | Commercial cell line | Lung | No | Evolutionary | Escalate dose | Yes | No | No |
| Katayma et al. 2012[111] | Targeted | Resistance mechanisms | Commercial cell line | Lung | No | Evolutionary | Escalate dose | Yes | Yes | Yes |
| Katayma et al. 2011[112] | Targeted | Treatment strategy | Commercial cell line | Lung | No | Evolutionary | Escalate dose | Yes | Yes | No |
| Engleman et al. 2007[40] | Targeted | Resistance mechanisms | Commercial cell line | Lung | No | Evolutionary | Escalate dose | Yes | No | No |
| Corcoran et al. 2010[25] | Targeted | Resistance mechanisms | Commercial cell line | Colon | No | Evolutionary | Escalate dose | Yes | Yes | No |
| Engleman et al. 2006[23] | Targeted | Resistance mechanisms | Commercial cell line | Lung | No | Evolutionary | Escalate dose | Yes | No | Yes |
| Ricci et al. 2002[39] | Targeted | Resistance mechanisms | Commercial cell line | Leukemia | No | Evolutionary | Escalate dose | Yes | Yes | Yes |
| Zhao et al. 2016[13] | Targeted | Collateral response | Commercial cell line | Leukemia | No | Evolutionary | Escalate dose | Yes | Yes | Yes |
| Bhang et al. 2015[113] | Targeted | Method | Commercial cell line | Lung | Yes | Clinical | Static dose | No | Yes | Yes |
| Scappini et al. 2004[114] | Targeted | Resistance mechanisms | Commercial cell line | Leukemia | No | Evolutionary | Escalate dose | Yes | No | Yes |
| Farrokhian et al. 2022[21] | Targeted | Ecology in cancer | Commercial cell line | Lung | No | Evolutionary | Static dose | Yes | Yes | Yes |
| Scarborough et al. 2020[7] | Chemo | Gene signatures, collateral response | Commercial cell line | Sarcoma | No | Evolutionary | Escalate dose | Yes | No | Yes |
| Lin-Rahardja et al. 2025[15] | Chemo | Gene signatures, collateral response, method | Commercial cell line | Sarcoma | No | Clinical | Static dose | Yes | No | Yes |
| Jado et al. 2024[42] | Chemo | Resistance mechanisms | Commercial cell line | Leukemia | No | Evolutionary | Escalate dose | Yes | Yes | No |
| Nishihara et al. 2022[26] | Targeted | Resistance mechanisms | Commercial cell line | Lung | No | Evolutionary | Escalate dose | Yes | Yes | Yes |
| Sharma et al. 2010[115] | Targeted | Drug tolerance | Commercial cell line | Lung | No | Evolutionary | Static dose | No | Yes | No |
| McDermott et al. 2010[27] | Targeted | Resistance mechanisms | Commercial cell line | Lung | No | Evolutionary | Escalate dose | NA | Yes | No |
| Hata et al. 2016[11] | Targeted | Resistance mechanisms, drug tolerance | Commercial cell line | Lung | Yes | Evolutionary | Escalate dose | Yes | Yes | Yes |
| Crystal et al. 2014[116] | Targeted | Drug discovery, treatment strategy | Patient derived cell line | Lung | No | Patient | NA | NA | Yes | No |
| Turke et al. 2010[43] | Targeted | Resistance mechanisms, treatment strategy | Commercial cell line | Lung | No | Evolutionary | Escalate dose | Yes | Yes | No |
| Kuwahara et al. 2017[117] | Radiation | Method | Commercial cell line | Liver | No | Clinical | Escalate dose | No | No | No |
| Grigoreva et al. 2021[118] | Chemo | Resistance mechanisms | Commercial cell line | Colon | No | Evolutionary | Escalate dose | Yes | No | Yes |
| Whiting et al. 2025[41] | Chemo | Resistance mechanisms | Commercial cell line | Colon | Yes | Evolutionary | Static dose | Yes | No | Yes |
| Acar et al. 2020[8] | Targeted | Collateral response, ecology in cancer | Commercial cell line | Lung | Yes | Evolutionary | Static dose | No | Yes | Yes |
| Zhang et al. 2022[119] | Targeted | Resistance mechanisms, drug discovery | Commercial cell line | Melanoma | Yes | Evolutionary | Static dose | Yes | Yes | Yes |
| Gutierrez et al. 2021[95] | Chemo | Ecology in cancer, method | Commercial cell line | Leukemia | Yes | Evolutionary | Static dose | No | Yes | Yes |
| Pfeifer et al. 2024[120] | Targeted | Resistance mechanisms | Commercial cell line | Lung | No | Evolutionary | Static dose | Yes | Yes | Yes |
| Hoare et al. 2022[121] | Chemo | Resistance mechanisms | Commercial cell line | Ovarian | No | Evolutionary | Escalate dose | Yes | Yes | Yes |
| Vander et al. 2020[9] | Targeted | Resistance mechanisms, collateral response | Commercial cell line | Lung | Yes | Evolutionary | Escalate dose | Yes | Yes | Yes |
| França et al. 2024[30] | Targeted | Resistance mechanisms, drug discovery | Commercial cell line | Ovarian, melanoma, lung | No | Evolutionary | Static dose | Yes | Yes | Yes |

**Table 2 – continued from previous page**

| Paper | Treatment | Objective | System | Cancer type | Barcoding | Drug dosing strategy | Drug escalation | Passaging | DNA | RNA |
|---|---|---|---|---|---|---|---|---|---|---|
| Tegze et al. 2012[122] | Chemo | Resistance mechanisms | Commercial cell line | Breast | No | Evolutionary | Escalate dose | Yes | No | Yes |
| Tsuruo et al. 1983[123] | Chemo | Resistance mechanisms, collateral response | Commercial cell line | Leukemia | No | Evolutionary | Escalate dose | NA | No | No |
| Han et al. 2014[124] | Chemo | Method | Commercial cell line | Sarcoma | No | Evolutionary | Escalate dose | No | No | Yes |
| Kimiya et al. 1992[125] | Chemo | Method | Commercial cell line | Pancreatic | No | Evolutionary | Escalate dose | Yes | No | No |
| Wattanawongdon et al. 2015[126] | Chemo | Method | Commercial cell line | Cholangiocarcinoma | No | Evolutionary | Escalate dose | No | No | Yes |
| Hur et al. 2016[127] | Chemo | Resistance mechanisms, method | Commercial cell line | Leukemia | No | Evolutionary | Escalate dose | Yes | Yes | Yes |
| Ohnoshi et al. 1982[128] | Chemo | Resistance mechanisms | Commercial cell line | Leukemia | No | Evolutionary | Escalate dose | No | No | No |
| Saijo et al. 1989[129] | Chemo | Method | Commercial cell line | Leukemia | No | Evolutionary | Escalate dose | NA | No | No |
| Harker et al. 1985[130] | Chemo | Collateral response, method | Commercial cell line | Sarcoma | No | Evolutionary | Escalate dose | Yes | No | No |
| Wirth et al. 2022[131] | Chemo | Method | Animal | Leukemia | Yes | Clinical | Escalate dose | NA | Yes | Yes |
| Machioka et al. 2018[132] | Chemo | Resistance mechanisms | Commercial cell line | Prostate | No | Evolutionary | Escalate dose | Yes | Yes | No |
| Takeda et al. 2007[133] | Chemo | Resistance mechanisms | Commercial cell line | Prostate | No | Evolutionary | Escalate dose | Yes | Yes | No |
| Megid et al. 2025[134] | Targeted | Resistance mechanisms | Commercial cell line | Lung | No | Evolutionary | Escalate dose | Yes | No | Yes |
| Gomes et al. 2022[135] | Targeted | Predictive biomarker | Commercial cell line | Head and neck | No | Evolutionary | Escalate dose | Yes | Yes | Yes |
| Chan et al. 2023[136] | Targeted | Resistance mechanisms | Commercial cell line | Lung | No | Evolutionary | Escalate dose | Yes | Yes | Yes |
| Williams et al. 2025[105] | Patient | Method, resistance mechanisms | Human/clinical | Ovarian | No | Patient | NA | NA | Yes | No |
| Danisik et al. 2023[24] | Chemo | Collateral response, treatment strategy | Commercial cell line | Colon | Yes | Evolutionary | Escalate dose | Yes | Yes | Yes |
| Chattopadhyay et al. 2025[137] | Nutrients | Evolution in cancer | Commercial cell line | Neuroblastoma | No | NA | NA | No | Yes | No |
| Al Bakir et al. 2025[138] | Patient | Resistance mechanisms | Human/clinical | Lung | No | Patient | NA | NA | Yes | No |
| Criscione et al. 2022[139] | Targeted | Collateral response, resistance mechanisms | Commercial cell line | Lung | No | Evolutionary | Static dose | Yes | No | Yes |
| Liu et al. 2011[140] | Chemo | Resistance mechanisms | Patient derived cell line | Cholangiocarcinoma | No | Evolutionary | Escalate dose | Yes | No | Yes |
| Němcová-Fürstová et al. 2016[141] | Chemo | Drug tolerance, resistance mechanisms | Commercial cell line | Breast | No | Evolutionary | Escalate dose | Yes | No | Yes |
| Bjornberg et al. 2026[22] | Targeted | Resistance mechanisms, evolution in cancer | Commercial cell line | Lung | Yes | Evolutionary | Static dose | NA | Yes | Yes |
| Chmielecki et al. 2011[142] | Targeted | Treatment strategy | Commercial cell line | Lung | No | Evolutionary | Escalate dose | Yes | Yes | No |
| Ogino et al. 2007[143] | Targeted | Resistance mechanisms | Commercial cell line | Lung | No | Evolutionary | Escalate dose | Yes | Yes | Yes |
| Godin-Heymann et al. 2008[144] | Targeted | Resistance mechanisms | Commercial cell line | Lung | No | Clinical | Static dose | Yes | No | No |
| Yang et al. 2026[145] | Targeted | Collateral response | Commercial cell line | Lung | No | Evolutionary | Escalate dose | Yes | No | No |
| Kono et al. 2026[146] | Chemo | Collateral response | Commercial cell line | Lung | No | Evolutionary | Escalate dose | Yes | No | Yes |
| Aldonza et al. 2020[147] | Targeted | Collateral response | Commercial cell line | Lung | No | Evolutionary | Escalate dose | Yes | No | Yes |
| Shien et al. 2013[148] | Targeted | Collateral response | Commercial cell line | Lung | No | Evolutionary | Escalate dose, static dose | Yes | Yes | Yes |