In Vitro and in Vivo toxicity Determination for Drug Discovery

Drugs Toxicology

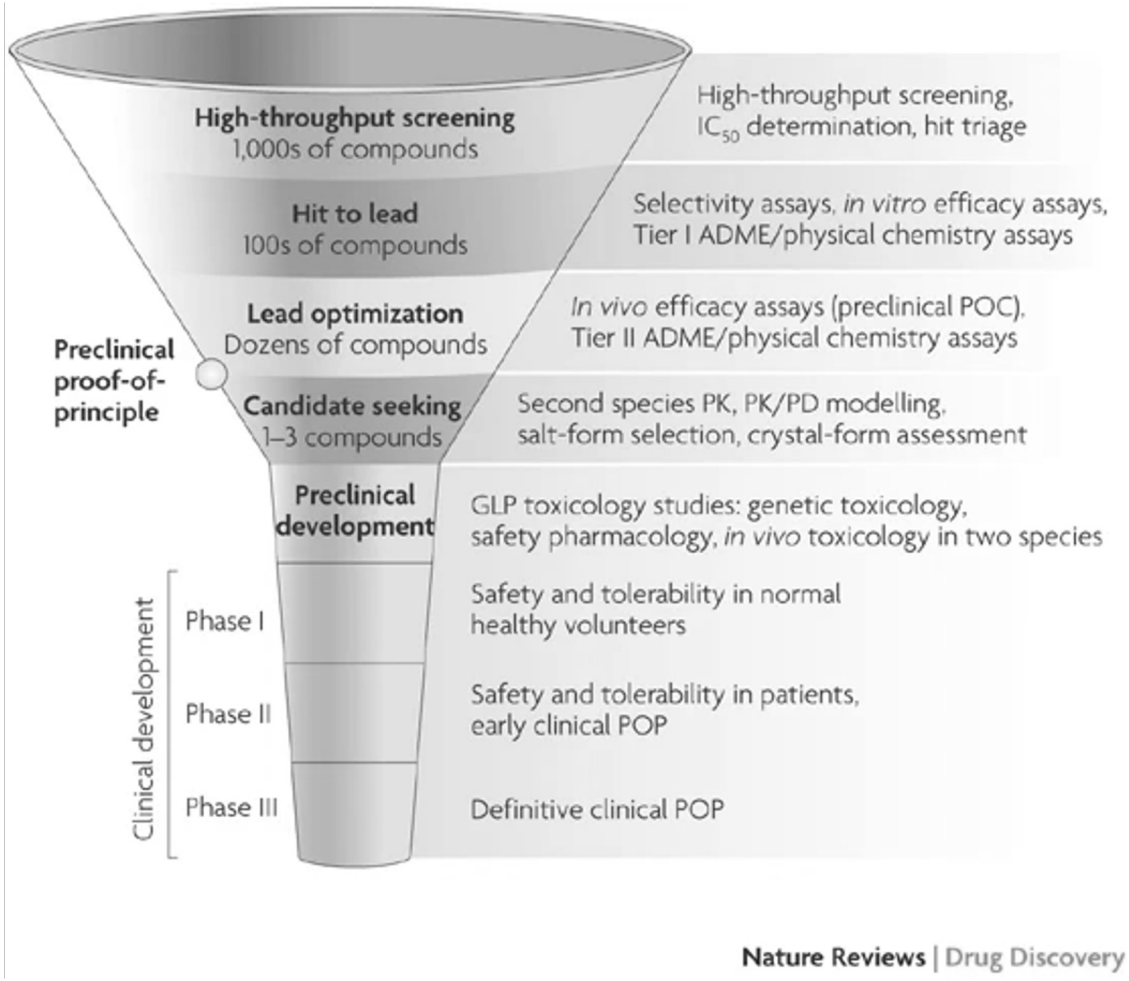
- The well-known “Paracelsus doctrine” states that the difference between a toxic and harmless compound is the dose.
- It is a general principle of toxicology that all compounds are toxic at some dose or exposure level, and an adequate safety margin defined *in vivo* is key to advancing a compound.
- Unfortunately, the dose–toxicity relationship is not always linear and depends critically on absorption, distribution, metabolism, and elimination characteristics of the drug. The term “toxicokinetics” is used to describe methods for relating drug dose to exposure levels and correlating both to development of toxicity indicators. Today, toxicity is a leading cause of attrition at all stages of drug development.
- Preclinical toxicities can be divided into three broad categories: primary pharmacology, secondary pharmacology and chemically mediated toxicity

Drugs Toxicology

Field	Definition
GENETIC TOXICOLOGY	Incorporates molecular biology principles in applications of toxicological sciences
OCCUPATIONAL TOXICOLOGY	Examines hazards associated with toxic exposure in the workplace
IN VITRO TOXICOLOGY	Development of cell culture and biochemical techniques to toxicity testing
ANALYTICAL TOXICOLOGY	Chemical and biochemical methods associated with identification and analysis of toxic substances
DEVELOPMENTAL TOXICOLOGY	Study of toxic substances and their effects on biological reproduction
IMMUNOTOXICOLOGY	Study of toxic substances and their effects on immunity
NEUROTOXICOLOGY	Study of toxic substances and their effects on nervous system

Primary pharmacodynamics	Studies on the mode of action and/or effects of a substance in relation to its desired therapeutic target.
Secondary pharmacodynamic	Studies on the mode of action and/or effects of a substance not related to its desired therapeutic target
Safety pharmacology	Studies the potential undesirable effects of on physiological functions in relation to exposure in the therapeutic range

Testing scheme for a small-molecule therapeutic



Focused libraries

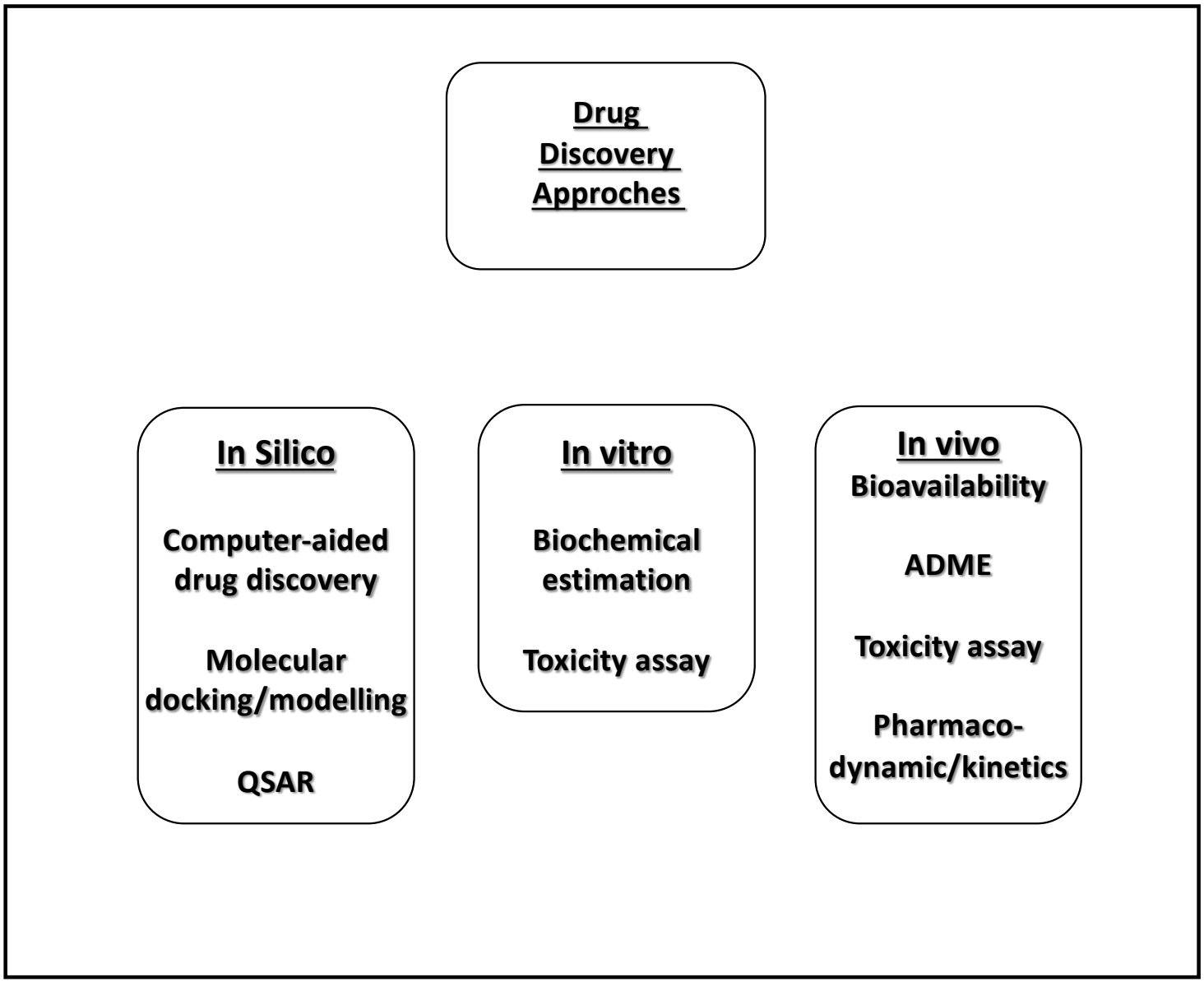
The primary goal of the high-throughput screening stage is to identify chemical hits from which lead series may be derived.

ADME assay

Potent and selective leads are then evaluated in early ADME (absorption, distribution, metabolism and excretion) assays

TOX assay

Compounds that successfully meet preclinical efficacy, ADME, pharmacokinetics and safety criteria are nominated as candidates for formal development.

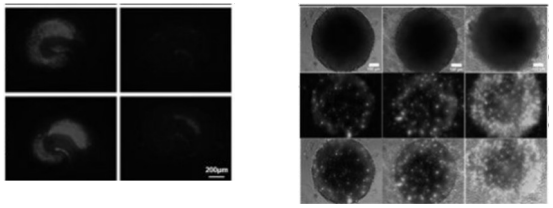

Drug Discovery Approches
In Silico
Computer-aided drug discovery
Molecular docking/modelling
QSAR
In vitro
Biochemical estimation
Toxicity assay
In vivo
Bioavailability
ADME
Toxicity assay
Pharmaco-dynamic/kinetics

Cytotoxicity Assays

Vital Dye

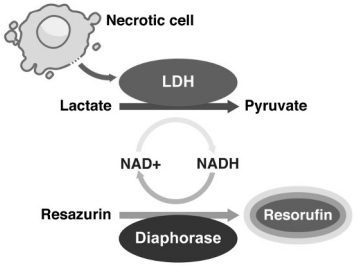
measure general cell **viability** or cell **proliferation** and are not specific to toxicity mechanisms.

Vital dyes, which are fluorescent or colored molecules, distinguish **between living and dead cells**



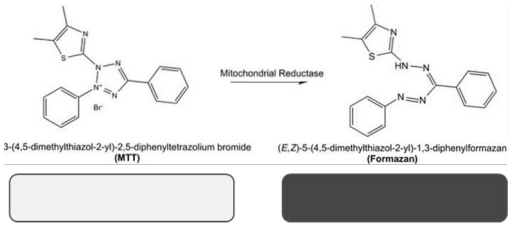
Intracellular protein release-based

measure the **leakage of cellular components** from compromised cultured cells when membrane integrity is altered, and especially measurement of intracellular proteins (most often enzymes) **in cell culture supernatants**



Metabolism based

measure NAD(P)H-dependent **oxidoreductase enzymes** which are present in viable cells and reduce the MTT to formazan or the **ATP** amount from cultured cells which is a **valid marker of viable cells**

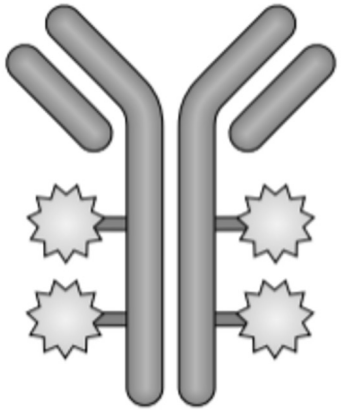


Common in vitro assays used in drug toxicity assessments

Assay name	Mechanism	Assessments	Use
MTT	Reduction of MTT to formazan	Measurement of anti-metabolic effects.	cell viability and proliferation
Cell Titer Blue	Reduction of resazurin to resorufin	Measurement of anti-metabolic effects.	cell viability and proliferation
CellTiterGlow	ATP quantification	Measurement of energy depletion effects	cell viability and proliferation
Lactate dehydrogenase (LDH) assay	Pyruvate conversion coupled to formazan	Measurement of cytolysis.	cell death
Live/dead Dyes	Dye uptake or exclusion by live cells.	Measurement of membrane integrity	Cell death
Caspase assays	Caspases are quantified using profluorescent substrates	Measurement of caspases activity	Apoptosis measurement

Antibody–drug conjugates (ADCs)

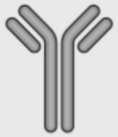
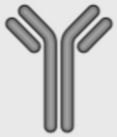
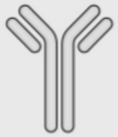
Antibody–drug conjugates (ADCs) are among the fastest growing drug classes in oncology. The mechanism of action of ADCsis complex, often requiring drug internalization followed by intracellular processing and payload release.



monoclonal antibodies (mAbs)

linker

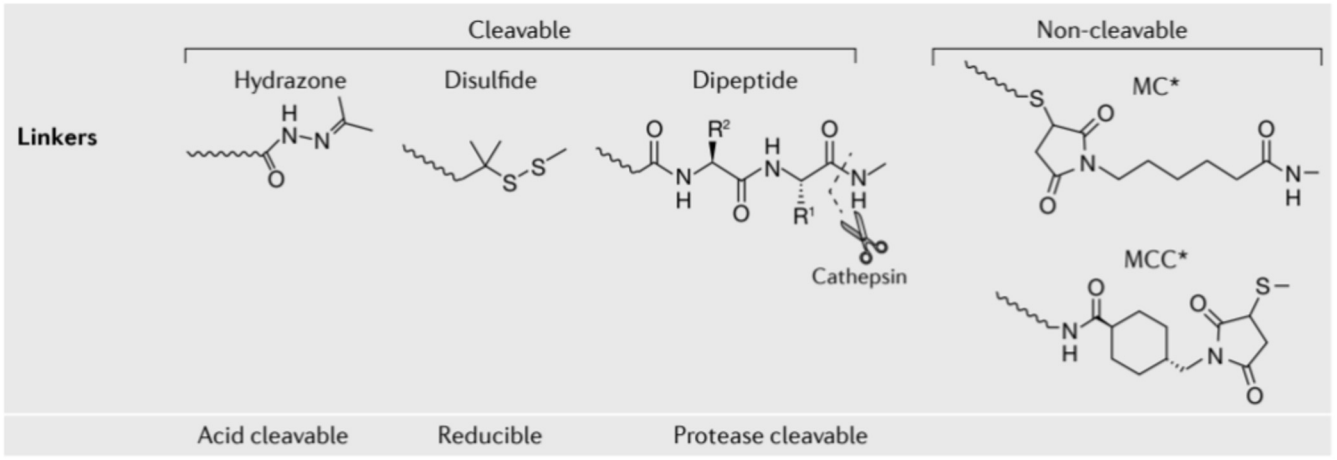
cytotoxic drugs (payload)

	IgG1	IgG2	IgG3	IgG4
Antibodies				
Serum half-life	21 days	21 days	7–21 days	21 days
C1q binding	Yes	Yes	Yes	No
Fcγ avidity	High	Low	High	Moderate

Although **antibody fragments** and bispecific antibodies present exciting opportunities for innovation, **immunoglobulin G (IgG)** remains the predominant antibody backbone used in this broad class of therapeutics as well as in ADCs specifically2

Human IgGs comprise four subclasses (IgG1, IgG2, IgG3 and IgG4), which differ in their constant domains and hinge regions. Subtle variations between these subclasses affect the **solubility and half-life of mAbs** as well as their affinity for different Fcγ receptors (FcγRs) expressed on immune effector cells

ADCs are designed to deliver their toxic payload to any cell expressing the target antigen and, thus, targets that are preferentially expressed in tumours versus non-malignant tissues present a wider therapeutic window and decrease the chance of systemic toxicities



The purpose of the linker is twofold. The first role is to ensure that the **cytotoxic payload remains firmly attached** to the antibody moiety **while the drug circulates in plasma**. Linkers that are unstable in plasma could release the payload prematurely, resulting in excess systemic toxicity and reduced payload delivery upon antigen engagement at the tumour site.

The second, often competing role of the linker is **to enable efficient release of the payload** within the tumour, particularly within cancer cells.

The choice of linkers

Cleavable linkers are designed to break down and release the cytotoxic payload of the ADC in response to tumour-associated factors such as acidic or reducing conditions or abundant proteolytic enzymes (for example, cathepsins). Examples of linkers cleaved by these mechanisms include:

pH-sensitive linkers

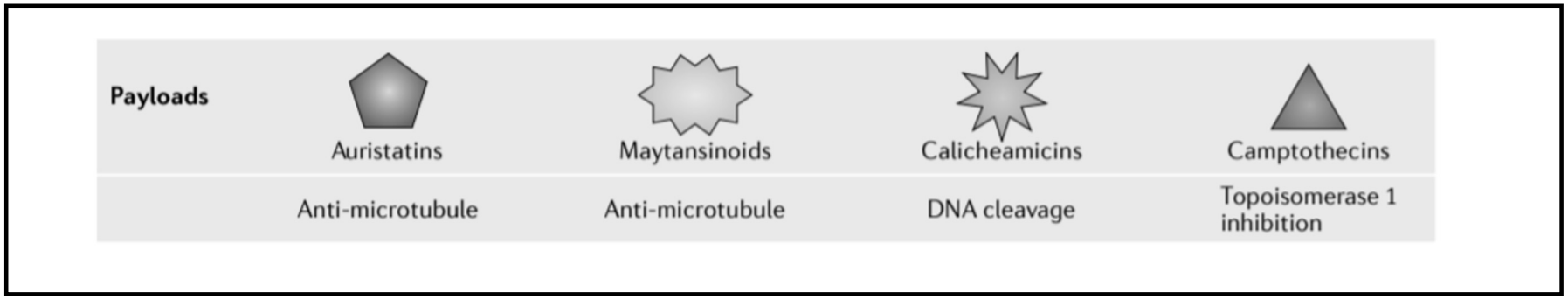
reducible disulfide linkers

peptide-based, enzyme-cleavable linkers

Non-cleavable linkers tend to be more stable in plasma but rely on lysosomal degradation of the entire antibody–linker construct to release their payloads, often resulting in the retention of charged amino acids on the payload, which might affect its action or cell permeability. Examples of non-cleavable linkers include:

thioether linkers

maleimide-based linkers



Early ADCs were designed to carry traditional **chemotherapy drugs** with known anticancer activity, such as methotrexate, doxorubicin or vinca alkaloids.

Microtubule inhibitors

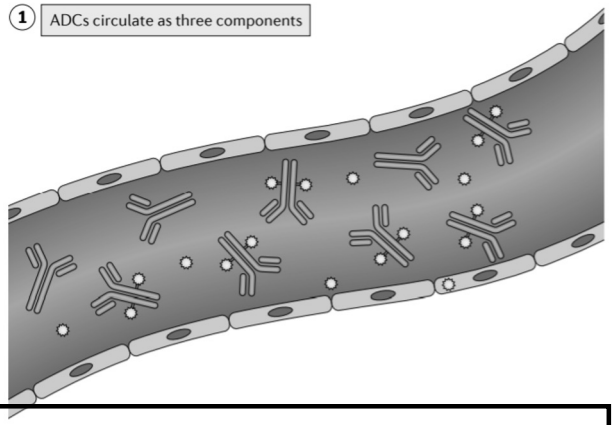
- Suristatins (MMAE)
- Maytansines (DM1/DM4)

DNA-Damaging agents

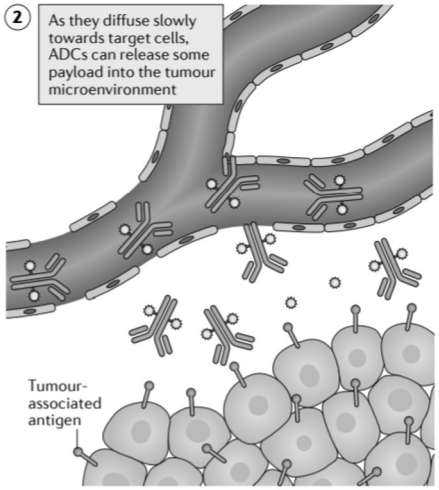
- Calicheamicin
- Antrhracyclines
- Duocarmycins
- Pyrrolobrnzodiazepines

The drug-to-antibody ratio (DAR) is the average number of payload moieties attached to each mAb. This property, which varies between ADCs, has implications for drug pharmacology and activity . DARs of currently approved ADCs range from 2 to 8

How ADCs work in vivo



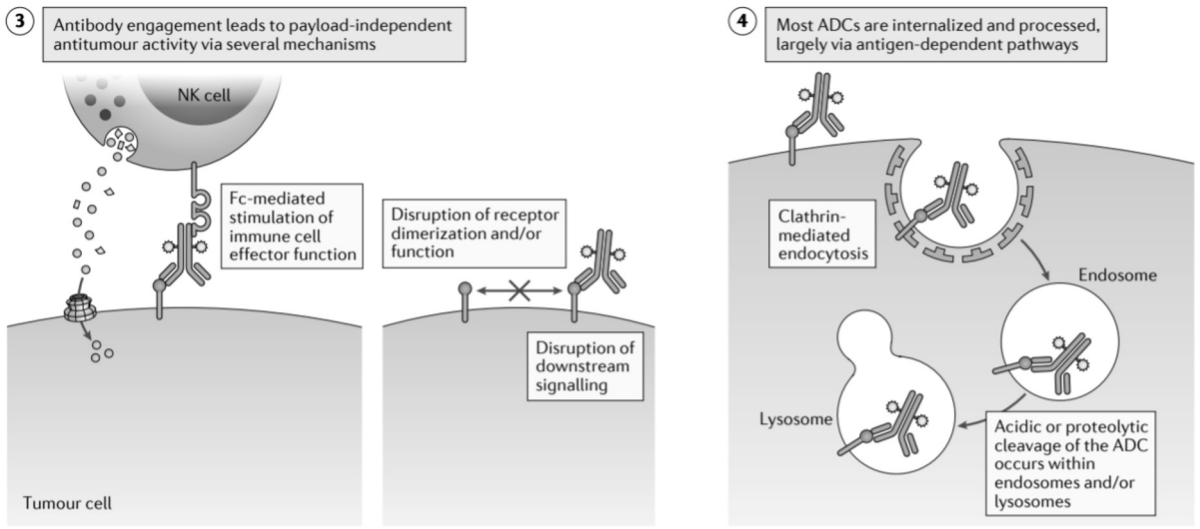
Upon administration, the ADC formulation contains three major circulating components: **the conjugate** (which constitutes the overwhelming fraction), **naked antibodies** and **free payload molecules**. These findings suggest that ADCs exist in vivo as a **dynamic admixture of circulating components**, which complicates pharmacological modelling and influences the clinical properties of these agents



In contrast to traditional cytotoxic therapies, mAbs are **large molecules**, a characteristic that limits their delivery to tumours. After extravasation from capillaries, antibodies reach tumour cells via passive diffusion, often resulting in slow, inefficient and heterogeneous tissue penetration

The **canonical model of ADC action** posits the following: binding of the mAb to the target antigen, subsequent internalization and, finally, linker breakdown and intracellular payload release. While this model serves as a helpful overall framework, the reality is more complicated and differs appreciably between ADCs.

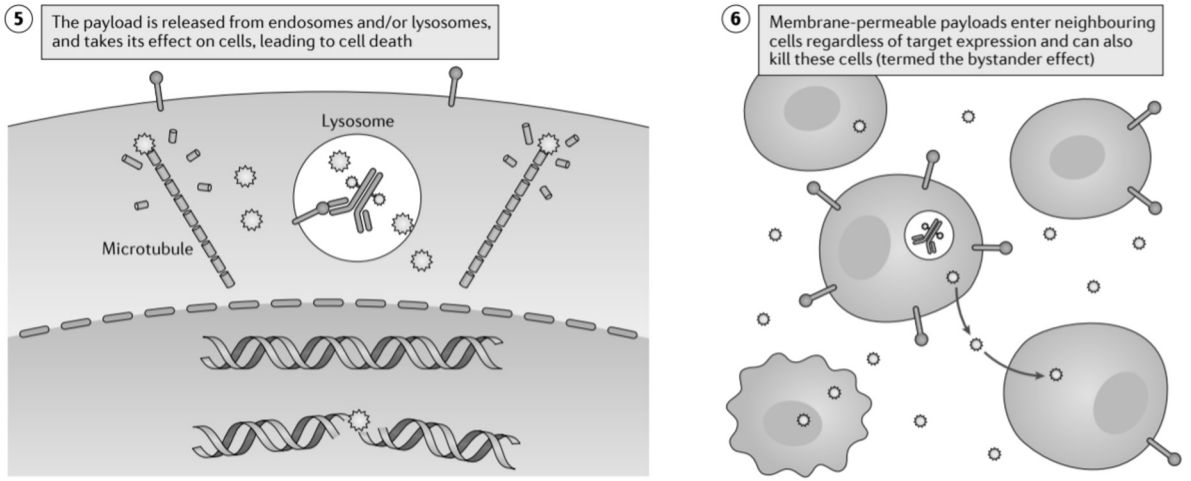
How ADCs work in vivo



Following tissue penetration, ADCs must engage with their target antigen for optimal cytotoxicity. Owing to the placement of linkers outside of the antigen-recognition domain of the mAb, ADCs typically bind to their target antigen with the same affinity as their unconjugated counterparts

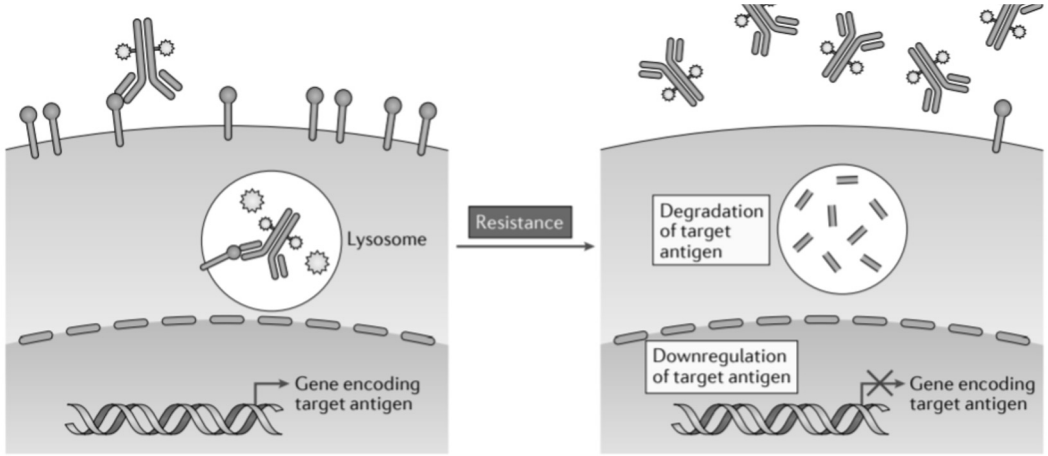
After antigen binding, the internalization of the ADC–antigen complex is thought to be a crucial step in payload delivery for many ADCs. **ADC internalization** can occur via the antigen-dependent processes of endocytosis or the antigen-independent process of pinocytosis, with clathrin-mediated endocytosis being the predominant mode of uptake. Following internalization, ADC–antigen complexes are trafficked along the endosomal and/or lysosomal pathways in a manner that seems to depend on proper organelle acidification

How ADCs work in vivo



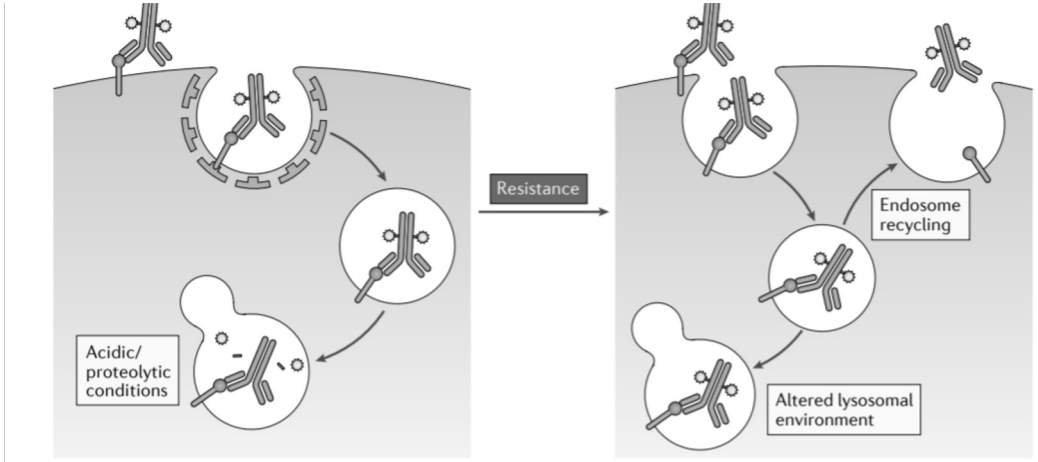
Payloads that are attached using acid-cleavable linkers are likely to be released in early endosomes, and those attached using linkers that are designed to be cleaved enzymatically or degraded via proteolysis are released in late endosomes or lysosomes. Reducible linkers release the payload principally upon exposure to glutathione, which is found at higher concentrations intracellularly than in plasma. The time from antigen engagement to terminal processing and payload release can be >24 hours. ADCs are designed to release their payload inside tumour cells in this **'Trojan horse' fashion.** Regardless of the compartment in which the payload is released, certain ADCs are capable of exerting a 'bystander effect' on neighbouring cells, irrespective of target antigen expression. For internalized ADCs, this property requires the diffusion of lipophilic payloads across cell membranes and is thought to be a major component of ADC activity against tumours with heterogeneous expression of the target antigen

Resistance to ADCs



ANTIGEN DOWNREGULATION OR LOSS

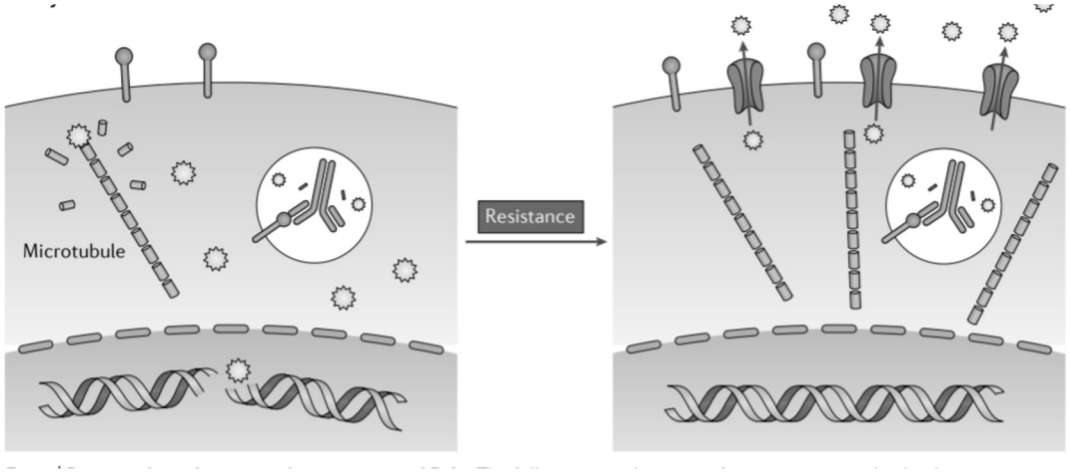
Downregulation of the target antigen by tumour cells can prevent ADCs from docking on tumour cells, thus reducing the release of the payload therein



ALTERATION OF INTRACELLULAR TRAFFICKING OR LYSOSOMIAL DRUG BREAKDOWN

Recycling of endosomes to the cell surface might result in ejection of the ADC back to the exterior of tumour cells prior to payload release; the alteration of lysosomal acidification, redox environment or proteolytic processes might also prevent adequate payload release.

Resistance to ADCs



PAYLOAD TOLLERANCY OR EFFLUX

The upregulation of ATP-binding cassette (ABC) transporter proteins in tumour cells can result in the active efflux of payload, thereby protecting cells from cytotoxic damage; however, not all payloads are ABC substrates.

FUTURE DIRECTIONS FOR ADC

Trials of antibody–drug conjugates in combination with other anticancer therapies are ongoing.

Increasing ADC delivery to tumour tissue

Antiangiogenic agents, such as those targeting the VEGF signalling pathway, might modify tumour vasculature in a way that improves ADC delivery to tumour tissues or enhances the cytotoxic effects of ADCs.

Modulation of antibody target expression and/or processing

Drugs that increase the cell-surface expression of the target antigen on tumour cells might promote antibody–antigen engagement. Alternatively, drugs that augment antigen turnover or degradation might promote ADC uptake and payload cleavage and release, thereby enhancing cytotoxicity. The conjugation with specific molecules targeting specific membrane lipid cellular compartments can enhance the cytotoxic effects of ADCs.

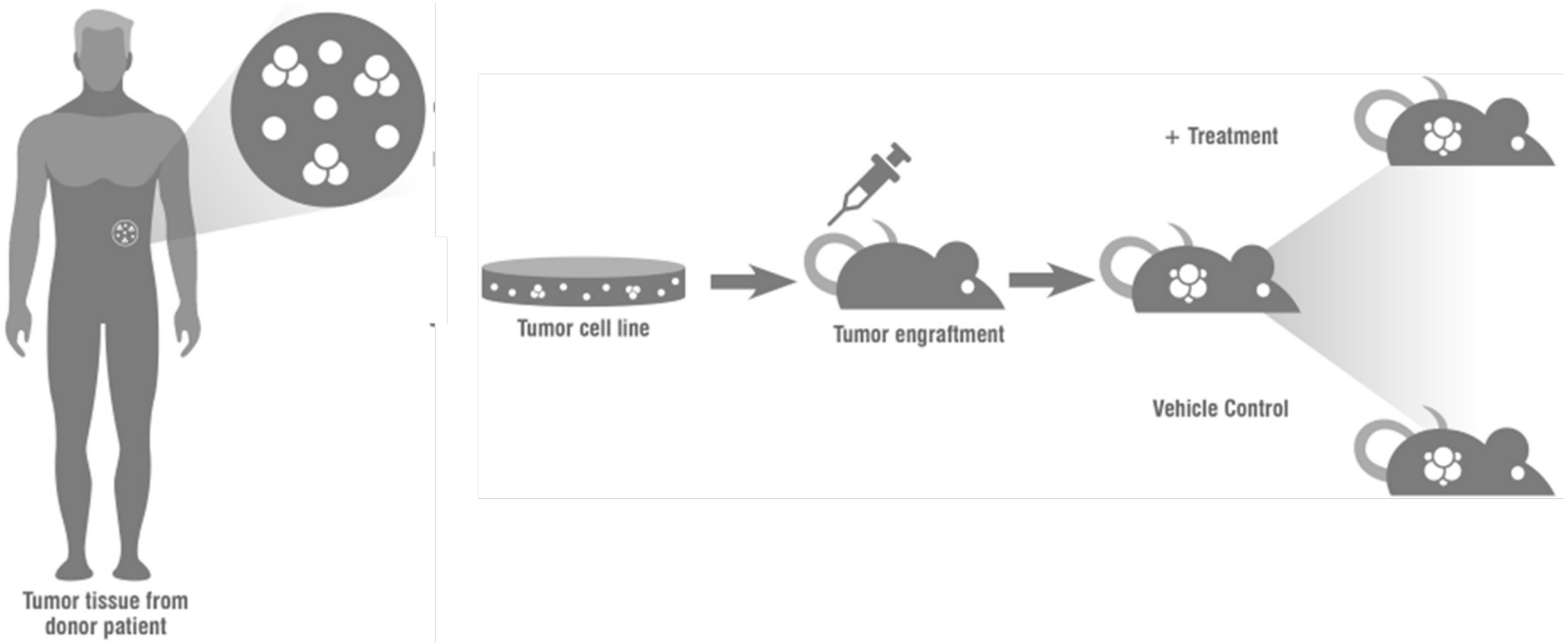
Potentiating payload activity and/or synthetic lethality

Payload activity can be potentiated with other agents that act synergistically through complementary mechanisms or synthetic lethality.

Promoting antitumour immunity

Immunotherapies have the potential to build on the antitumour immunity induced by ADCs, either by enhancing antibody-dependent cellular cytotoxicity or by augmenting cell-mediated tumour recognition and immune effector function.

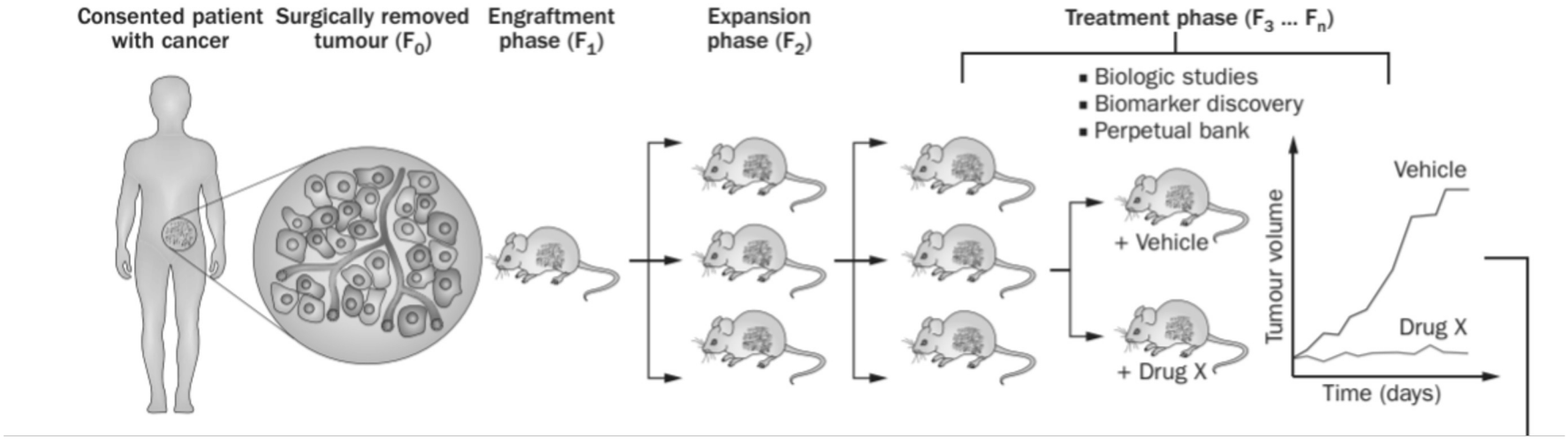
Patient-derived xenografts for oncology drug development



Cell-line derived xenograft (CDX).
Cells from a human cancer cell line or from tumour sample are injected into the subcutaneous space or other sites in the mouse to form a tumour.

Cell-line-derived xenograft (CDX) models have provided valuable information that has improved our understanding of cancer development and the mechanisms of drug actions

Patient-derived xenografts for oncology drug development

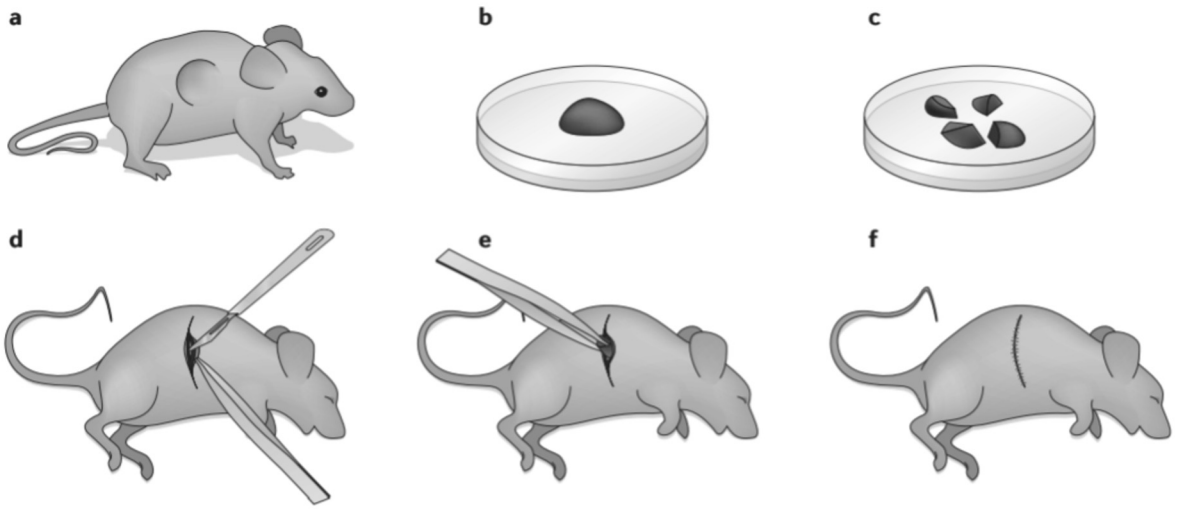


Patient-derived xenograft (PDX)

Human tumour tissue is directly transplanted into the subcutaneous space or other sites in the mouse without employing cell lines grown on tissue culture plastic.

The approach is very straightforward, consisting of obtaining fresh surgical tissue, sectioning it into ~3mm^3 pieces, followed by subcutaneous or orthotopic implantation into the flank of an immunodeficient mouse or rat

Subcutaneous transplantation of patient-derived xenografts



Tumour tissues are grown subcutaneously on the back of immunodeficient (nude) mice.
Tissue is extracted from the tumour at the time of scarification. Tumour tissues are cut into which are placed subcutaneously using forceps

PDX VERSUS CDX

Advantages

PDX vs CDX

Direct engraftment from human tumour

Preserved tumour heterogeneity and lineage hierarchy and conserved contribution of human stromal cells (early passages)

Shortcomings

PDX vs CDX
Increased cost

Transplantation site is usually limited to subcutaneous tissue

Difficulty in quantitative engraftment

Reduced efficiency of gene manipulation with regards to: exogenous gene expression; gene silencing; luciferase expression for in vivo imaging

xenografts
for oncology drug development

- C/PDX tumours maintain the molecular, genetic and histological heterogeneity typical of tumours of origin

- The tumour histology of C/PDX models provides an excellent *in vivo* preclinical platform to study cancer stem-cell biology and stromal–tumour interactions; novel cancer therapeutics can also be assessed

- Well-characterized C/PDX models represent an information-rich preclinical resource for analysis of drug activity, including novel–novel drug combinations, as well as predictive biomarker discovery

Immunodeficient Mouse model

Nude mice (nu/nu).

The first strains used for the study of human cancer. **They lack functional T cells**, but the **presence of intact B** cells and an intact innate immune system, including **natural killer** (NK) cells, impairs efficient engraftment of primary solid human tumours.

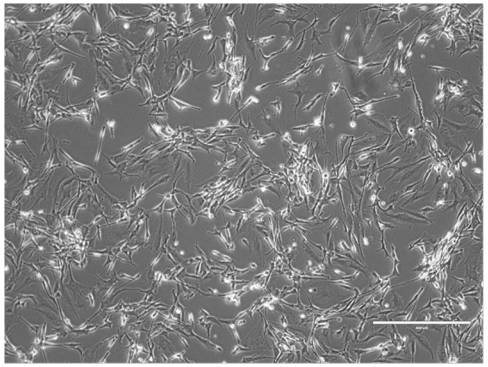
Scid mice.

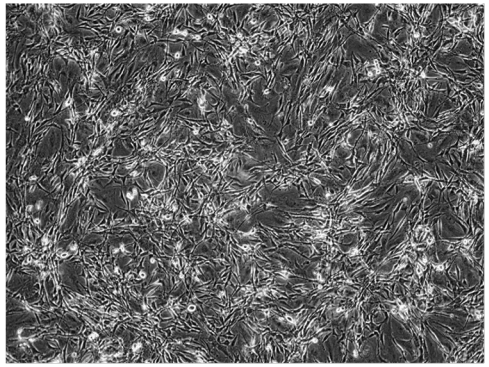
Mice with severe combined immunodeficiency (scid) aided the establishment of patient-derived xenografts (PDXs), which were developed in C.B17 mice. The term scid now represents all severely immunodeficient strains of mice. The mutation predominantly **prevents the development of mature T cells and B cells** and scid strains are more suitable for engraftment of primary solid human tumours than nude mice. However, mature T cells and B cells develop in some C.B17-scid mice when **they get old** and they have an intact innate immune system, including moderate NK cell activity, which reduces take rate and growth of primary solid human tumours.

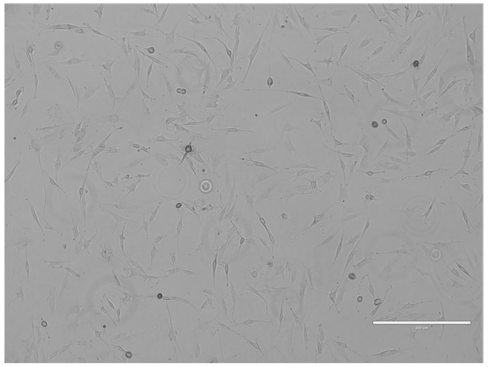
NOD scid mice.

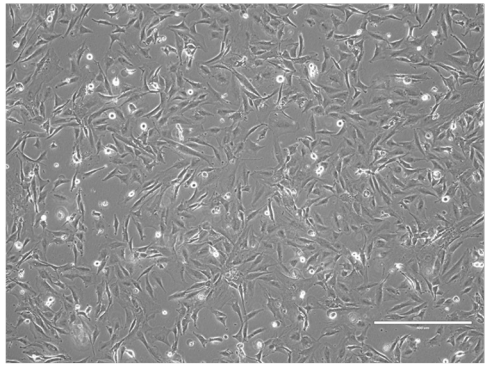
The nonobese diabetic (NOD) strain has an impairment in **innate immunity, including reduced NK cell and macrophage activity, abnormal dendritic cell development, and lack of haemolytic complemen**t. Backcrossing the scid mutation onto NOD strain mice led to mouse host (NOD scid mice) being more receptive of primary solid human tumours than nude or scid mice. The shortcomings of this strain include the development of thymic lymphomas by 8–9 months old and a short lifespan (~8.5 months).

Primary culture of glioblastoma (GBM) cell lines





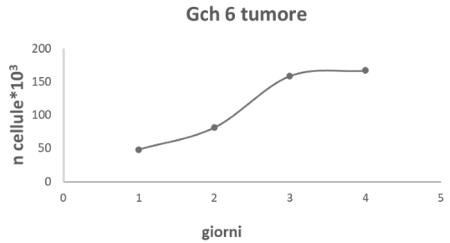




bassa confluenza

alta confluenza

Gch 6 tumore



Gch 6 peritumore

Tumorigenic