Integrating cellular and tissue dynamics with cell fate decisions through computational modeling

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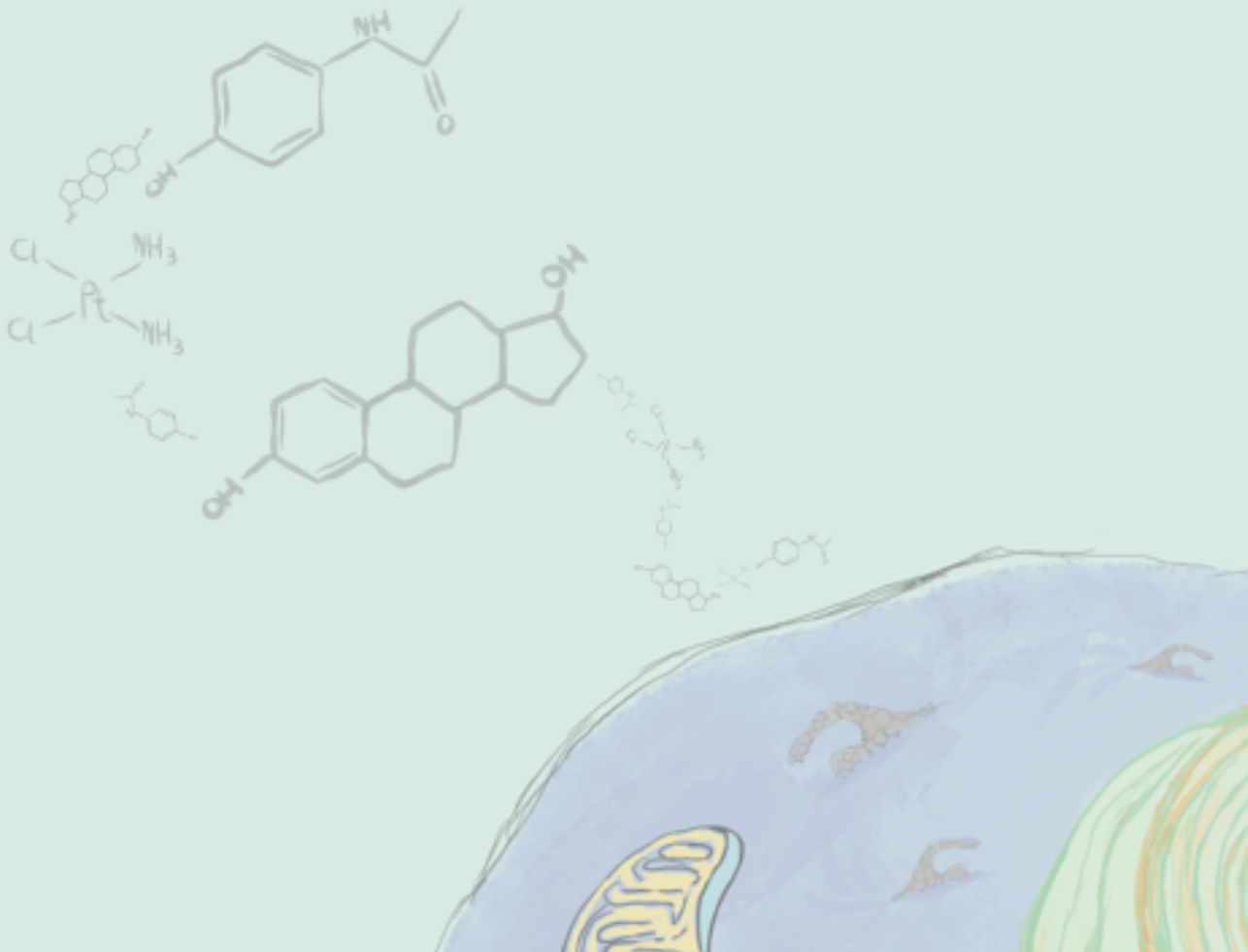
Chapter 1

Introduction



Introduction

Animal testing remains a necessary step during the preclinical phase of drug development. However, the drug failure rate in clinical testing phases I, II and III is approximately 90%, which can be partly ascribed to unmanageable toxicity [1]. Moreover, toxicity studies in animals are poor predictors for human adverse effects, with congruence rates of around 50% between humans and animals, and among animal species being commonly observed [2]. Finding alternatives to animal testing has been a topic of research since the 1960s, when the principle of the 3Rs was introduced: replacement, reduction and refinement of animal experimentation [3]. *In vitro* research methods with human cells are already widely used for preclinical toxicity testing and new approaches are being developed to improve their predictive capacity for healthy human tissue. In addition, there is an ever increasing interest in computational methods to assist hazard and risk assessment, with a more than 10 fold increase in the number of papers using *in silico* modeling to support safety predictions published in the years between 1990 and 2015 [4]. The mechanistic insight gained with modeling is essential for our understanding of adverse effects and useful for the prediction of adversity for novel compounds. In this thesis, we investigate the link between intracellular molecular processes and adverse effects using mathematical modeling. In this way, we couple phenomena that occur on different levels of biological organization, which generates hypotheses about the mechanisms behind adversity and provides openings for future experimental testing as well as approaches to predict adversity without animal experiments.

Chemical-induced disruption of homeostasis

Besides understanding the pharmacokinetics of a drug that describes the process of absorption, distribution, metabolism and excretion (ADME), it is essential to gain insight in its pharmacodynamics, i.e., the intracellular signaling pathways and cellular activation cascades at tissue level (e.g., recruitment of specific immune cells) that are activated upon exposure. Moreover, to unravel the mechanisms behind adverse effects, we need to understand how signaling cascades regulate cell fate. Under normal conditions, healthy cells are in homeostasis, i.e., the concentration of intracellular biomolecules such as proteins do not change over time or do so in a highly regulated manner (e.g., circadian rhythms). Upon chemical-induced molecular stress, cells have to adapt to the new conditions, repair the damage inflicted by the chemicals and eliminate toxicants. To achieve this, cells activate stress response pathways that are typically regulated by one or a few central transcription factors. Depending on the type and severity of stress, distinct transcription factors are activated, each of which induces the transcription of its own downstream targets. This gene activation results in protein expression dynamics that are specific for the exposure scenario. Among the most well-studied chemical-induced stress response pathways are the oxidative stress response (OSR), unfolded protein response (UPR), inflammatory stress response (ISR) and DNA damage response (DDR). Several proteins that cells start to express upon activation of these

pathways are involved in the regulation of cell fate, such as an increased or decreased level of proliferation, and different forms of cell death.

High stress levels can lead to irreversible adverse effects, some of which are highly dependent on protein signaling cascades, e.g., apoptosis and senescence, whereas others are considered to be largely passive processes such as necrosis. In contrast, low and intermediate levels of stress usually lead to an adaptive stress response, in which cells temporarily change their normal behavior to overcome chemical insults. Cell cycle arrest, that can occur among others upon oxidative [5–7], unfolded protein [8] and DNA damage stress [9,10], permits a cell to resolve damage before committing to mitosis, such that cell progeny remains uninjured. Regulation of cell cycle progression often involves activation of the transcription factor p53, encoded by the *TP53* gene. This protein is central to the DDR, but is also associated to other pathways. For example, cell cycle arrest as a consequence of protein misfolding is p53-dependent [11]. In addition, the OSR and DDR pathways interact closely: p53 can induce the production of reactive oxygen species (ROS) [12,13], and vice versa, oxidative stress can result in DNA damage and activation of p53 [14,15]. Although novel drugs are rarely genotoxic because this would immediately disqualify them as an appropriate medicine, the hub function of p53 makes this protein an important subject for studying adverse effects.

Underlining the central function of p53 in cell homeostasis is the large number of genes it regulates. p53 has more than 300 downstream targets of which many are involved in responses such as cell cycle arrest, DNA repair, metabolism and apoptosis [16]. Some of these targets can be used as markers for cell fate. The well-known p53 target *CDKN1A*/p21 is an important driver of transient and permanent cell cycle arrest and therefore serves as one of the hallmarks of senescence [17,18]. Yet, complex processes such as cell cycle regulation are typically not coordinated by a single protein, but by a complex system of interacting biomolecules, and must thus be studied as such. In addition, parenchymal cells are subject to extracellular factors such as damage-associated molecular patterns (DAMPs), senescence-associated secretory phenotype (SASP), immune-cell derived mitogens and cytokines, that can all modulate cell responses. Mitogens stimulate cell cycle progression and mitosis [19,20], whereas the release of SASP can induce paracrine senescence in neighboring cells [21,22]. Thus, to understand the processes underlying cell fate determination, both intra- and intercellular dynamics should be explored.

Unraveling protein dynamics of stress response pathways

Human cell lines are still widely used in well-established 2D culture methods for high-throughput drug testing as well as in advanced methods using organ-like cell systems such as organs-on-a-chip and organoids. Cells from an immortalized cell line can proliferate indefinitely and are therefore easy-to-use *in vitro* systems for experimentation. Cell lines can represent a wide variety of

different cell types, which depends on their original source. For example, there are cell lines that originate from liver tissue, such as the hepatocellular carcinoma cell line HepG2 or hepatic progenitor cell line HepaRG, from kidney tissue (e.g., RPTEC), or from breast cancer tissue (e.g., MCF7). Because the liver is the primary organ responsible for drug metabolization, this organ is especially sensitive to drug-induced injury [23,24]. Indeed, drug-induced liver injury (DILI) is prevalent during clinical trials as well as after drug approval [25]. Therefore, testing for hepatotoxicity in a liver-originating cell line such as HepG2 could be particularly relevant to assure drug safety.

Continued developments in *in vitro* methodology and technology greatly augment the volume and quality of data used to study the effects of drugs on an organism. Instead of blotting, for which cells need to be lysed, the use of confocal microscopy in combination with fluorescent protein tagging allows one to measure protein expression in living cells. With this live-cell imaging technique, a population of cells can be monitored over time which permits one to study drug effects over time on a single-cell level. The relative abundance of proteins over time in response to drug exposure can be measured with a broad panel of stress response HepG2 BAC-GFP reporter cells [26]. Because each signaling pathway typically has one or more central regulators, activation of these proteins indicated by an increase in intensity of the fluorescent signal indicates the occurrence of a specific type of molecular stress. For example, cisplatin and etoposide, two chemotherapeutic agents that serve as positive control for the activation of the DDR, indeed activate p53, as well as its downstream targets MDM2, p21 and BTG2 [27]. Similarly, diethyl maleate (DEM) activates SRXN1 via activation of the OSR transcription factor NRF2 [27].

Despite the practicality of cell lines for culturing and experimentation, they do not respond in exactly the same manner as cells in healthy tissue. Cell lines are isolated from the body and therefore lack the influence of other cell types and interactions with surrounding cells. In addition, cell lines often have a distinct gene and protein expression pattern which distinguishes them from healthy cells [28,29]. Instead, primary human hepatocytes (PHHs) can function as an appropriate cell system representing healthy human liver tissue due to their high expression of enzymes and transporters [30]. The potential divergence in sensitivity to drug exposure in various cell types is well-illustrated by the response to cisplatin and acetaminophen (APAP) in HepG2 and PHHs. Both HepG2 cells and PHHs elicit the DDR upon cisplatin exposure. However, APAP, a compound that is known to elicit oxidative stress in liver tissue [31], does not cause activation of NRF2 in HepG2 cells [27], whereas PHHs do elicit the OSR upon APAP exposure [32]. However, PHHs are currently not suitable for long-term culture, which complicates the study of protein dynamics and cell fate determination in these cells. Thus, it depends on the research question which biological *in vitro* cell system is best suited to use for experimentation.

Impact of chemical exposure on cell fate

To understand how intracellular protein dynamics relate to cell fate determination, we first need to elucidate cell behavior during homeostasis. In homeostatic conditions, many biochemical reactions in the cell are in steady state, i.e., the protein concentrations stay approximately constant over time. However, cell cycle progression is a cyclic process, which implies that proteins involved in cell cycle progression are only on average in steady state. The fluctuations in protein abundance during a single cycle make the cell cycle a complex subject of study. A cycling cell goes through four distinct cell cycle phases. At the start of the cycle, cells are in growth or gap phase 1 (G1). Once cells commit to proliferation, they replicate their DNA in the DNA synthesis phase (S). The S phase is followed by gap phase 2 (G2), during which cells are preparing and committing to division in the mitotic phase (M). The main drivers of cell cycle progression are cyclins and cyclin-dependent kinases (CDKs). The abundance of cyclins changes during cell cycle progression [33]. Cyclins C and D are abundant during G1 phase and E-type cyclins are highly expressed during S phase. In addition, cyclins A and B accumulate in S phase, after which cyclin A peaks in G2 and cyclin B in M phase. CDKs accomplish their enzymatic activity and promote transitions throughout the cell cycle by binding to these cyclins [33,34]. Apart from cyclin/CDK complexes, many other proteins are involved in cell cycle regulation, some of which play a role in chemical-induced changes during cell cycle progression. Such chemical-induced effects can either be inhibitory, i.e., leading to a slowing down of cell cycle progression and potentially even completely block cell cycle arrest, or stimulatory, i.e., leading to fast cell cycle progression and potentially to cancer induction.

The first possibility (disturbance of normal cell behavior and induction of cell cycle arrest) can occur at intermediate stress intensities, for example resulting from DNA damage. Cyclin-dependent kinase inhibitors (CKIs) target CDKs or cyclin/CDK complexes and thereby interfere with normal cell cycle progression. The p21 protein, that is induced upon DNA damage, functions as CKI, as it binds multiple cyclin/CDK complexes and induces a G1 cell cycle arrest [35–38]. Cell cycle inhibition induced by p21 is dependent on binding of retinoblastoma protein (RB) and E2F transcription factor complex [38]: due to the inactivation of cyclin/CDKs by p21, RB becomes hypo-phosphorylated and binds to E2F. This RB/E2F complex subsequently binds to the DNA and thereby prevents gene transcription, which causes cell cycle arrest. E2F transcriptionally regulates the expression of many genes involved in cell cycle progression, among which are Geminin and Cdt1 [39]. Geminin is inhibited by Anaphase Promoting Complex/Cyclosome (APC/C), which is another core component of the cell cycle responsible for continuation of the G1/S transition and entry into S phase[40,41]. APC/C is a multimeric ubiquitin E3-ligase that controls the degradation of multiple cyclins and other proteins involved in the cell cycle [42].

The second possibility (promotion of cell cycle progression) is frequently induced by steroid hormones and endocrine disrupting chemicals (EDCs). EDCs are present in many products encountered in everyday life, such as food, pesticides and cosmetics, and can bind hormone receptors which causes interference with normal cell cycling. Disruption of endocrine signaling by EDCs can cause developmental problems and has been associated with an increased risk to develop cancer, reviewed in [43]. One of the important hormone receptors affected by EDCs is the estrogen receptor alpha (ERα). Binding of a ligand to this receptor causes transcriptional activation of downstream targets involved in cell cycle progression, cell growth and survival, and cellular maintenance [44], as well as downregulation of targets involved in cell cycle arrest [45]. Growth-regulating estrogen receptor binding 1 (GREB1) is a critical regulator of cell cycle progression upon activation of ERα by 17β-estradiol (E2) [46]. Two other ERα targets, progesterone receptor (PR) and trefoil factor 1 (TFF1), have also been associated to cell cycle progression, although the underlying mechanism remains to be elucidated [47]. Therefore, how ERα signaling affects cell cycle progression is an ongoing topic of research.

In contrast to low concentrations of chemical compounds that typically lead to reversible cell fates such as the discussed temporary cell cycle arrest, high concentrations can cause stress to the degree that irreversible cell fates arise, such as necrosis, apoptosis and senescence. Senescence is characterized as a permanent and irreversible cell cycle arrest, that occurs in severely damaged cells to prevent propagation of damaged cells and thereby suppress tumorigenesis [48]. In senescent cells, the CDK inhibitors p21 and p16 accumulate and activate RB, which causes inactivation of E2F and subsequent cell cycle arrest [49]. The transcription factor p53 is responsible for induction of p21 and is therefore one of the triggers of senescence. However, p53 is also one of the primary regulators of apoptosis and induces the production of pro-apoptotic proteins such as p53 upregulated modulator of apoptosis (PUMA), apoptosis regulator BAX and Bcl-2 homologous antagonist/killer (BAK1). The choice between induction of the different cell fates depends on the modulation of DNA binding by p53 through DNA sequence and chromatin structure, and post-translational modifications, interactions with co-factors, expression dynamics and oligomerization state of p53 [50,51]. This highlights once again the complexity of the regulation of cell fate by intracellular signaling cascades.

Integrating different levels of biological organization

To achieve a quantitative coupling of protein expression to cell fate, experimental information about high-level cell behavior is needed to complement protein expression data. There are many methods to experimentally determine cell cycle progression and cell death. A well-established method to demonstrate cell proliferation uses the incorporation of thymidine analogues BrdU or EdU during DNA replication in S phase, and thus indicates cell cycling [52]. Because this method depends on visualization with

antibodies using immunohistochemistry or immunofluorescence, it requires fixation of the cells prior to imaging, which prevents one from monitoring cell cycle progression over time in single cells. In contrast, measuring DNA content with time-lapse flow cytometry does allow live-cell monitoring over time [53], yet this method determines cell cycle progression for a population of cells and can therefore not be used to track long-term dynamics in individual cells. Alternatively, high-content confocal microscopy readouts based on fluorescent staining of cells can be used to determine cell cycle progression. For example, the expression of fluorescently labelled p21 protein has been used as marker for cell cycle progression in single cells [54]. This method requires single cell tracks (i.e., a sequence of images that captures at least two phases of the cell cycle in a single cell) that visualize mitotic events and p21 dynamics in both control conditions and in exposure scenarios to determine cell cycle phase. In contrast, fluorescent labelling of Geminin and Cdt1 in the fluorescent ubiquitination-based cell cycle indicator (FUCCI) technology allows cell cycle determination for cells in a single image, because Geminin and Cdt1 are expressed during distinct phases of the cell cycle [55]. In addition, with sufficient time resolution and image quality, the expression of these marker proteins and thus cell cycle progression can be monitored over time in individual living cells.

Similar to the large number of methods to monitor cell cycle progression, there are numerous assays to measure cell viability. These include generic assays that measure cell death regardless of the type of death, such as the detection of release of lactate dehydrogenase (LDH) enzymes into the culture medium, but there are also methods that specifically detect apoptosis, autophagy or necrosis [56]. Live-cell imaging is also a powerful tool to monitor cell death occurrences in a single population of cells over time. For this purpose, propidium iodide (PI) or Annexin V (AnV) can be added to the medium and fluorescently stain cells upon their death [56].

To unravel the mechanism underlying cell fate determination, solely measuring protein expression and determining cell fate is not sufficient. In addition, due to the vast number of proteins involved in cell fate regulation and the complexity of their interactions, it is difficult to discern the key determinants of cell fate. Simplification of such a biological system with mathematical modeling can help to unravel the core actors necessary to explain cell fates like cell cycle progression or arrest. Different types of mathematical models can be used to create an *in silico* representation of a biological process and it depends on the biological question which model type is most suited to answer it. In drug safety testing, creating an adverse outcome pathway (AOP) is a commonly used method to describe a sequence of biological events that occur at different levels of biological organization within a single conceptual framework. In this context, the applicability of quantitative methods is highest when they naturally integrate with other models, to create a single computational model that simulates the effect of drug exposure on molecular and systems level. A

frequently used modeling method in biochemistry is ordinary differential equation (ODE) based modeling. ODE models describe the temporal dynamics of variables, such as chemical compounds and proteins, or the number of cells in a certain state. Separate ODE models can be easily coupled, which makes these models highly suitable for modeling biological processes of different types and are therefore one of the preferred tools for quantitative AOPs [57]. Another appropriate method to integrate molecular dynamics with cell outcome is provided by the cellular Potts model (CPM) [58,59]. A CPM can be used to model cells and tissues on a 2D or 3D lattice while accommodating diversity in characteristics such as cell size and shape amongst cell types. The CPM model formalism can incorporate intracellular protein dynamics, thereby providing a natural way to integrate molecular signaling with cell fate. Thus, a CPM is an accessible method to combine multiple biological processes in a single model, yet it is key not to create an overly complicated model. An optimal model describes real-life observations by focusing on essential processes and disregarding insignificant mechanisms. As such, it provides a framework to generate testable hypotheses that further advance our understanding of the mechanisms underlying biological phenomena like cell fate decisions.

Thesis outline

In this thesis, we study how stress pathway activation is linked to cell fate. By means of computational models, we describe various intracellular protein signaling cascades and couple the expression dynamics to cell outcomes such as transient cell cycle arrest, cell cycle progression, senescence and necrosis.

First, we study the activation of the DNA damage response upon cisplatin-induced DNA damage in HepG2 cells by creating an ODE model that describes the activation of *TP53*/p53 and its downstream targets *MDM2*/MDM2, *CDKN1A*/p21 and *BTG2*/BTG2 on mRNA and protein level (**Chapter 2**). To investigate whether this HepG2-based model can be used to predict pathway activation in PHHs, we exploit this model to create simulations of protein dynamics in virtual PHH sample donors, and compare the correlations between the expression of *TP53* and its downstream targets in the virtual donors to the correlations found in experimentally determined PHH data. The predicted positive *TP53-CDKN1A* and *TP53-BTG2* correlations based on model simulations agree with the correlations in PHHs, but the slightly negative *TP53-MDM2* correlation in PHHs cannot be reproduced with our model. Although the model fits the data of the protein dynamics very well, this incongruence between the model-based virtual donors and PHHs indicates that either the cisplatin-induced DDR dynamics in HepG2 cells deviate from the dynamics in PHHs, or that our model is missing essential interactions to explain the experimentally observed negative relation between *TP53* and *MDM2* expression.

Next, we investigate whether the DDR protein dynamics can explain the cisplatin- and etoposide-induced cell cycle arrest that occurs for exposure to

pre-cytotoxic concentrations of these compounds (**Chapter 3**). For this purpose, we utilize the DDR model proposed in Chapter 2, couple it to a cell cycle model that simulates the number of cells in each cell cycle phase, and calibrated the model parameters to time-resolved data of the number of HepG2-FUCCI cells in each phase. These cell population-level data exhibit an accumulation of cells in S-G2-M phase already at low doses of the chemotherapeutic agents, but also a continuation of the cell cycle to G1 phase at late time points. With our model, we show that BTG2 expression alone is sufficient to explain the cisplatin-induced, but not the etoposide-induced G2 arrest. In addition, the detailed dynamics of the subsequent cell cycle continuation cannot be explained in full by p21 or by BTG2, because the expression of these proteins remains high at late time points. This highlights the complexity of cell cycle regulation and the requirement of model extensions to predict (temporary) cell cycle arrest.

Following our study of cell cycle arrest on cell population level, we examine the estrogen response pathway and its connection to cell cycle progression, this time on single cell level (**Chapter 4**). This requires a novel approach, in which we couple estrogen-induced protein signaling of ERα, GREB1 and PR to an elementary cell cycle model previously constructed by Ferrell *et al*. (2011) [60] and subsequently exploited by Bae *et al*. (2019) [61] to determine cell cycle phase durations. To evaluate the accuracy of our model, we experimentally determine cell phase durations for individual cells. For this purpose, we use live-cell imaging of MCF7-FUCCI cells and develop a convolutional neural network for high-quality cell segmentation, and subsequent single cell tracking. Our model successfully predicts the G1 arrest that follows PR knockdown and the prolonged S-phase that is a result of GREB1 knockdown.

Finally, we expand our single-cell approach to liver tissue level to gain insight in the interplay between intra- and intercellular factors that drive cell fate (**Chapter 5**). We construct a spatial model that simulates the distribution, uptake, metabolism, and excretion of APAP. Our model describes cellular behaviors at low and high APAP concentrations in a single liver lobule and the consequent liver damage and - if possible - recovery. In this theoretical model, we use intracellular DDR signaling with p21 as most important readout as determinant for the induction of senescence, whereas immune cells have a dual role by promoting senescence on the one hand, but by clearing senescent cells and by stimulating recovery through the release of mitogenic biomolecules. With this model, we investigate these counteracting stimulants for senescence and proliferation, and demonstrate the applicability of the model by investigating the effect of various therapies to APAP overdosing *in silico*.

In our final **Chapter 6**, we discuss how our models help to unravel intracellular protein dynamics as a consequence of chemical-induced stress and provide testable hypotheses for future exploration. In addition, we further highlight how modeling allows us to quantitatively integrate processes on different levels of

biological organization and provides insight into mechanisms that lead to adverse effects.

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