

REVIEW ARTICLE

Pseudolaric Acid B as a Promising Antitumor Natural Product with Context–Dependent Cell Death Mechanisms

Xinran Yang¹, Shuyu Huang¹, Yunfeng Xiao¹, Ying Hu², Dianbao Zhang^{1,*}, Fei Yu^{2,*}

ABSTRACT: Pseudolaric acid B (PAB), a diterpenoid natural product from *Pseudolarix amabilis*, has been shown to have a wide range of pharmacological effects. Although the chemical properties, structural modifications, and biological activities of PAB have been recently reviewed, how PAB induces apoptosis, ferroptosis, autophagy, senescence and mitotic catastrophe, and the implications from this context-dependent mechanism remain elusive. From a cell fate decision network viewpoint, the underlying mechanisms of PAB are explained here. We propose that PAB is a context-dependent cell death modulator rather than a multi-target cytotoxic agent. Five types of cell death are closely linked to each other via several important factors, namely, reactive oxygen species, p53, glucose metabolism, autophagy, and iron metabolism. We emphasize that ferroptosis is becoming a hotspot in PAB research. This is because PAB can simultaneously target several important nodes in the ferroptosis network by regulating up-regulation of transferrin receptors, activation of Nox4, suppression of xCT, activation of NCOA4-induced ferritinophagy, and degradation of GPX4. Moreover, the conversion of PAB from a multi-target natural product to precision-targeted degrader is discussed in detail, via the development of CD147-based PROTACs. Finally, how PAB can alter the tumor microenvironment, including angiogenesis, polarization of immune cells and regulation of gut microbiota, is discussed. Three key questions are proposed, i.e., how to determine which type of cell death PAB causes; should ferroptosis be considered as a main target for the development of PAB; and can the PROTAC strategy be applied to other natural products? In order to answer these questions, more evidence is required to confirm the cell fate decision network, and biomarkers should be identified to predict cell death, and PAB-based precision medicine should be developed.

Keywords: Pseudolaric acid B; Ferroptosis; Cell fate decision; PROTAC; Tumor microenvironment; Context-dependent cell death

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1. INTRODUCTION

Natural products are an excellent source of lead compounds, and one example is the diterpenoid Pseudolaric acid B (PAB), which can be isolated from the root bark of *Pseudolarix*

amabilis [1-3]. This compound displays pre-clinical antifungal, anticancer, anti-inflammatory and immunomodulatory properties [1, 2]. It was also discovered to have anti-tubulin polymerisation activity and G2/M phase cell cycle arrest, making it a promising anticancer candidate [3, 4]. There has been extensive research into PAB over the last two decades. You et al recently published a thorough review detailing the natural sources, isolation and extraction methods, synthesis, structure-activity relationships and bioactivities of PAB [1]. This review provided an overview of information available and highlighted areas where more research is needed; namely further research into the mechanism of action, pharmacokinetics-pharmacodynamics

¹ Department of Stem Cells and Regenerative Medicine, China Medical University, Shenyang 110122, China.

² Department of Traditional Chinese Medicine, Shengjing Hospital of China Medical University, Shenyang 110004, China.

* Authors to whom correspondence should be addressed:

dbzhang@cmu.edu.cn (D. Zhang)

yuf1@sj-hospital.org (F. Yu)

relationship and toxicology [1]. Previous reviews have also been published on the immunomodulatory and anticancer properties of PAB [2] as well as the isolation, bioactivities and synthesis [3]. However, a major question still remains. PAB causes different cell death pathways depending on the cell type. It causes caspase-mediated apoptosis in HeLa cervical cancer cells [5]. In A549 lung cancer cells PAB causes senescence via p53-p21 pathway [6, 7]. PAB induces autophagic cell death in H1975 non-small cell lung cancer cells via ROS/AMPK/mTOR pathway [8]. In glioma cells PAB causes ferroptosis via Nox4 induction and xCT suppression [9]. In fibrosarcoma L929 cells PAB mainly causes cytosolic autophagy [10, 11]. How can this compound cause these different effects, what does this mean for the development of a treatment?

This review presents a fresh perspective on PAB, moving beyond its classification as a multi-target cytotoxic agent with multiple biological functions. We propose that PAB induces death in cells via a complex network that includes all five major death pathways. Within this network we highlight the critical nodes that drive death fate decisions. PAB becomes a context specific inducer of cell death. This highlights the emergence of ferroptosis as an emerging hub within PAB pharmacology. Furthermore, we connect this knowledge to the broader theme of multi-target natural products evolving into highly targeted degrader agents using the CD147 based PROTAC system. This review provides a novel framework which links key mechanistic nodes to therapeutic applications. This framework can be used to inform discovery of biomarkers, patient stratification and rational combination treatment strategies. Additionally, this framework allows us to make predictions regarding the cellular context that PAB will drive death via specific death pathways.

We put forward a hypothesis that the PAB is a context-dependent cell death regulator rather than multi-target cytotoxic agent. First, we established a cell death decision-making network for the five major PAB-induced cell death modes and identified the key nodes in the network. Next, we focus on the ferroptosis, an emerging hotspot during PAB pharmacological studies. Then we discuss the transformation from multi-target natural product to precisely targeting degrader using PAB-based CD147 PROTAC as an example. Besides, we also discuss the role of PAB in reshaping the pathogenic microenvironment, including angiogenesis, polarization of immune cells and modulation of gut microbiota. Finally, we proposed three critical questions to guide the future investigation of PAB: 1) How to predict the cell death modes PAB may trigger? 2) Should we focus on ferroptosis during the development of PAB?, and 3) Can the PROTAC strategy be used for other natural products?

The network of cell fate decisions following PAB treatment stems from the fact that the 5 types of PAB-induced cell death are interlinked. Apoptosis, ferroptosis, autophagy, senescence, and mitotic catastrophe are interlinked through key factors such as reactive oxygen species, p53 status, glucose metabolism, autophagy, and iron homeostasis [7-11, 12-26]. PAB can be considered an input factor that leads to

different outcomes depending on the nature of the cells, including their genetic profile, and metabolic status. Understanding this network means that therapeutic effects caused by PAB cannot be predicted based on the PAB alone, and must be understood in relation to the genetic and metabolic status of the tumour. This also means that combinations of PAB and other agents should be designed to take into account this network.

Ferroptosis is an important pathway and PAB triggers many ferroptosis related pathways [9, 20-22]. PAB causes disruption to many points in the ferroptosis pathway unlike erastin, RSL3 and sorafenib. PAB causes disruption to iron uptake, iron storage, ROS production, glutathione biosynthesis and GPX4 activity [9, 20- 22]. This could explain its effectiveness against a wide variety of cancer cell types. However, this lack of specificity may be a concern. Ferroptosis can be very context dependent. This makes it a possible treatment option as PAB has been found to be selective to cancer cells in some situations [1, 22]. Understanding how PAB interacts with the ferroptosis pathway in different contexts will be key to developing it as a precise treatment.

Another highlight of this review is the transformation of a multi-targeting natural product into targeting degraders. PAB exhibits several protein binding interactions, including tubulin, CD147, FLT4, CDK1 and others [4, 27-34]. This can often be considered a benefit; however can cause issues with selectivity. In recent years PAB based PROTACs have been developed, specifically a CD147 PROTAC, which enables the transformation of PAB from a multi-targeting cytotoxic agent into a selective target degrader [32]. This would allow less off target effects and provide an improved therapeutic index. It would also allow investigation of individual targets within complex biological systems. The successes seen with the CD147 PROTAC suggest that similar strategies can be applied to other PAB targets; however challenges exist with chemical modifications, linkers and E3 ligases [32].

Thirdly, the capacity of PAB to reprogram the pathogenic microenvironment. PAB exhibits anti-angiogenic effect via several mechanisms: inhibition of endothelial cell proliferation and migration, suppression of hypoxia-induced VEGF secretion, and promotion of HIF-1 α degradation [35-38]. PAB can also polarize macrophages from M1 to M2 phenotype during inflammatory processes [39-42]. Additionally, PAB can regulate T-cell responses by inhibiting Th1/Th17 differentiation and promoting regulatory T cells [43-45]. In metabolic diseases, PAB can regulate gut microbiota and related metabolites [46-48]. These effects are not directly cytotoxic to tumour cells or pathogens, suggesting that PAB could be beneficial when used in combination with other microenvironment targeting therapeutics.

Herein, we describe a framework for cell fate determination networks that incorporates our knowledge of PAB mechanisms and highlights crucial nodes in the signaling pathways. The new focus on ferroptosis in the field of PAB pharmacology is presented. In addition, we explore the shift from multi-target natural products to specific

degraders using the CD147 based PROTAC approach. Finally, we investigate how PAB are able to reprogram the disease microenvironment. Three crucial questions were identified that future work should address. Furthermore, we explored how the framework can provide insights into biomarker identification, patient stratification and rational combination therapies. This review provides a comprehensive overview of our knowledge of PAB mechanisms and presents a new framework that can move us towards a predictive understanding. This reveals what experiments should be prioritized to fully realize the therapeutic potential of this natural product.

2. THE FATE DECISION NETWORK OF DEATH MODES

Pseudolaric acid B (PAB) can cause at least 5 different types of cell death or cell cycle arrest in cancer cells including apoptosis, ferroptosis, autophagy, senescence and mitotic catastrophe. However, these have been described as distinct processes with limited connection. Thus far this has only been of benefit for catalogue purposes but does not address the main issue as to how one drug can cause so many different effects. A more valuable way to think about this is to see these different effects as outputs of a network. This network contains several key nodes that integrate the PAB signal to decide which outcome occurs. We will discuss each type of cell death, the molecular machinery involved and the nodes that link them together below.

2.1. Apoptosis

Apoptosis is the best characterised form of cell death triggered by PAB. The mitochondrial pathway is heavily affected by PAB treatment, causing down-regulation of Bcl-2 and Bcl-xL. There is increased levels of pro-apoptotic proteins Bax and Bad, decreased mitochondrial membrane potential, cytochrome c release and caspase-9 and caspase-3 activation [12, 13, 14]. Some cell types also undergo apoptosis via the death receptor pathway. PAB induces death receptor 5 (DR5) and activates caspase-8 in head and neck cancer cells [15]. PAB-induced apoptosis is also partially dependent on JNK activation and phosphorylation of p53 in HeLa cells [5, 6].

PAB activates different upstream pathways to cause mitochondrial dysfunction in different cell types. PAB activates the AMPK/JNK/DRP1 pathway causing mitochondrial fission and apoptosis in hepatocellular carcinoma [17]. In triple negative breast cancer, PAB inhibits the PI3K/AKT/mTOR pathway to cause apoptosis [18]. PAB also induces apoptosis in gastric cancer cells through p53 dependent induction of p21 and up-regulation of Fas/APO-1 [19]. The differences in upstream pathways are an early sign that PAB will induce apoptosis dependant on the cell type due to its distinct signalling pathways. However, the variety of upstream signals leading to apoptosis via the mitochondrial

pathway suggest that Bcl-2 family members and caspase cascade are a universal pathway used to induce apoptosis when the correct upstream signals have been activated.

2.2. Ferroptosis

Ferroptosis is a form of programmed cell death involving iron-dependent lipid peroxidation. PAB has been shown to trigger ferroptosis [9]. PAB promotes ferritin receptor expression, enhances iron levels within the cell, activates Nox4 and suppresses the xCT/GSH pathway, causing lipid peroxidation to occur [9]. This cell death can be rescued by using deferoxamine or ferrostatin-1 [9]. This showed that lipid peroxidation occurs due to PAB, as using iron chelators and lipid peroxidation inhibitors can stop cell death.

PAB has also been shown to cause ferroptosis in various ways. PAB can increase ferritinophagy in lung adenocarcinoma cells by increasing NCOA4, leading to an increase in labile iron levels and lipid peroxidation [20]. In colon cancer cells, PAB derivatives can decrease GPX4 activity and increase proteasomal degradation [21]. In non-small cell lung cancer cells PAB causes ferroptosis via increased survivin by upregulation through JNK/ERK [22]. This shows that PAB can cause ferroptosis via multiple pathways. This shows that despite the different pathways involved in ferroptosis caused by PAB, they all lead to lipid peroxidation. This shows that ferroptosis is a clear programmed cell death pathway that can be triggered by PAB. PAB can trigger it in many ways, which is unusual for ferroptosis inducers. This will be discussed in more detail in section 3.

2.3. Autophagy

Autophagy plays a context-dependent role within PAB treated cells. In some cell lines, autophagy acts as an adaptive response to help prevent cell death. For example in MCF-7 breast cancer cells PAB causes upregulation of autophagy via the upregulation of Beclin-1 and LC3-II. When autophagy was inhibited using 3-methyladenine (3-MA) there was an increase in cell death [23]. However, in L929 fibrosarcoma cells PAB causes cytostatic autophagy, meaning autophagy does not cause apoptosis. The amount of autophagy will determine if senescence or apoptosis occurs [10, 11]. This means autophagy can act as an adaptive response to determine the cells fate.

However, in some cases autophagy can cause cell death. PAB can cause autophagy in H1975 and H1650 non-small cell lung cancer cells via the ROS/AMPK/mTOR pathway. Chloroquine in combination caused an increase in LC3-II and p62 but a decrease in apoptosis. [8]. In prostate cancer cells PAB caused both caspase-dependent apoptosis and autophagy-induced cell death [24]. Autophagy can have a double role within PAB treated cells. Overall, autophagy can act as an adaptive response or cause cell death. It is not clear how PAB causes autophagy to be either an adaptive or

cytotoxic process within the cells. The amount of time and intensity at which autophagy occurs, the availability of substrates to degrade and the health of the lysosomes will all play a role in determining the outcome. The context-dependent nature of autophagy is key to determining whether or not the cells will survive to become senescent or undergo apoptosis or ferroptosis.

PAB can induce senescence in several cancer cell lines. Prolonged exposure of A549 lung cancer cells to PAB results in G2/M arrest followed by mitotic catastrophe and senescence [7]. This has been found to be p53 dependent as senescence is suppressed when p53 is knocked down [7]. However, PAB can also cause senescence in H460 cells which have wild-type p53. In contrast, H1299 cells which are p53-null experience apoptosis rather than senescence when exposed to PAB [7]. The induction of senescence being p53 dependent highlights the role of p53 in senescence. However, also shows that p53 is an important decision point between senescence and apoptosis.

This decision between senescence and apoptosis is influenced by glucose metabolism. PAB induces increased glucose metabolism and senescence in A549 cells with wild-type p53 [25]. However, if glucose metabolism is inhibited either by 2-deoxyglucose, p53 knockdown or p21 knockdown then senescence is switched to apoptosis [25]. PAB also induces decreased glucose metabolism and apoptosis in H1299 cells which are p53-null [25]. This shows that glucose metabolism and p53 are important points in the decision making process. The senescence/apoptosis decision being dependent on glucose metabolism shows that the decision is not purely based on the apoptotic pathways but instead depends on the metabolic state of the cell. This allows

us to understand why there may be heterogeneity in responses to PAB due to heterogeneity in glucose metabolism in tumours.

2.5. Mitotic Catastrophe

PAB causes disruption of microtubules leading to mitotic catastrophe in various cell lines. In L929 cells PAB causes mitotic arrest and multinucleation [26]. Long term exposure results in inactivation of cyclin B1-cdc2 complexes causing mitotic slippage [26]. In A172 neuroglioma cells PAB causes changes in tubulin aggregation resulting in cell cycle slippage from G2/M to G0/G1 and then to S phase [27]. This agrees with PAB's known effect of destabilizing microtubules. Generally long term mitotic arrest will result in apoptotic cell death or mitotic slippage.

Mitotic catastrophe can result in multiple forms of cell death depending on treatment length and cell type. In L929 cells it can result in apoptosis, senescence or autophagy [26, 28, 29]. This supports the idea that death is mediated via a network. Mitotic catastrophe would be considered to occur at the top of the network resulting in creation of the environment needed for death via other mechanisms. Multinucleation, aneuploidy and proteotoxic stress would be created. Whether the cell would undergo cell cycle exit (and senescence) or start apoptotic or autophagic mechanisms would depend on how long the mitotic block was and how competent the spindle assembly checkpoint was. The network of the 5 different forms of cell death is shown below in Figure 1.

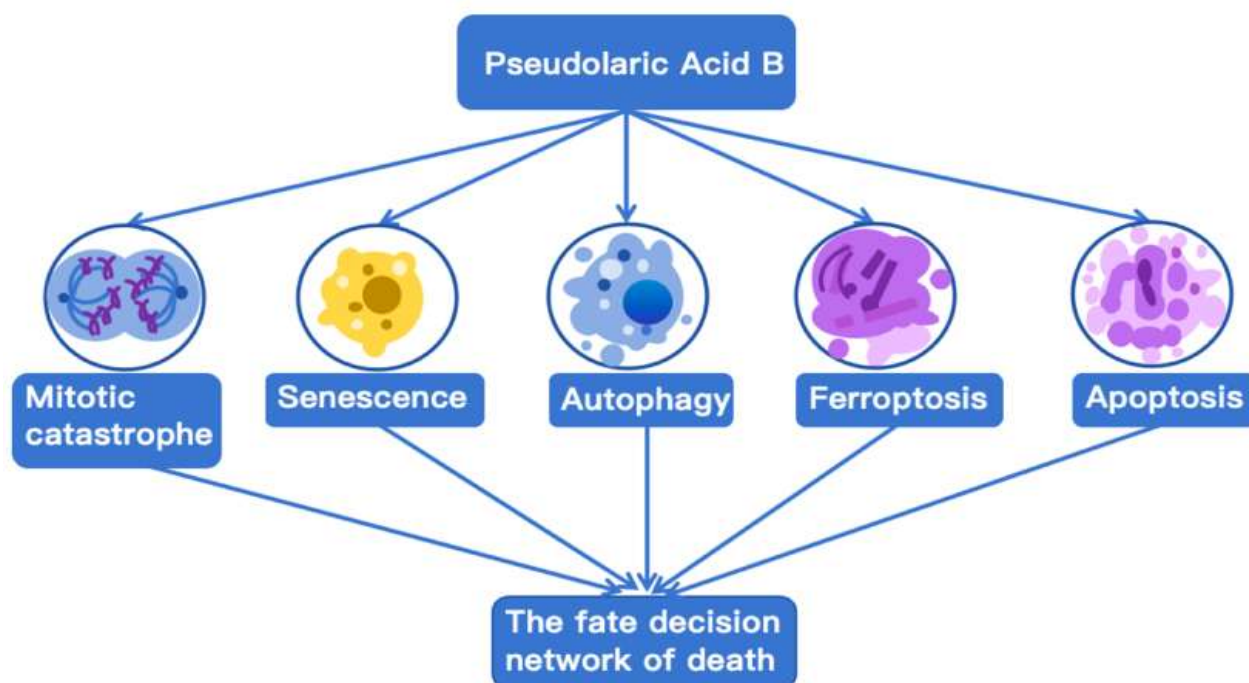


Fig. 1. The fate decision network of five death modes upon PAB treatment.

2.6. Key Nodes in the Decision Network

The death modes are not mutually exclusive and are linked via several key nodes. Firstly, reactive oxygen species (ROS) impact on multiple death modes. In non-small cell lung cancer cells, PAB increases ROS levels which activates AMPK/mTOR signaling to increase autophagy/apoptosis [8]. In L929 cells, PAB activates a positive feedback loop involving ROS/JNK/p53 to regulate autophagy-dependent senescence [29]. Finally, in glioma cells ROS produced via Nox4 activation contribute to ferroptosis [9]. This highlights the importance of ROS within the network and also explains why anti-oxidants can promote different death modes in response to PAB.

The presence of p53 impacts on the decision made. PAB tends to cause senescence in p53 wild type cells [7], however apoptosis occurs in p53 null cells [25]. Senescence is mediated through the p53/p21 pathway whereas p53 also regulates ferroptosis by controlling xCT [9]. This means p53 is a central hub within the network.

Glucose metabolism can drive the decision between senescence and apoptosis. Increased glucose metabolism causes increased senescence, whereas decreased glucose metabolism results in increased apoptosis [25]. This means the metabolic profile of PAB resistant cells (for example, if they rely on glycolysis) could be a good predictor. Autophagy can act as a protective mechanism or a death promoting mechanism depending on the level and duration. Some cells undergo senescence due to autophagy [29] whereas others experience delayed apoptosis [10]. This makes autophagy context dependent meaning that there isn't a clear positive or negative impact on the network.

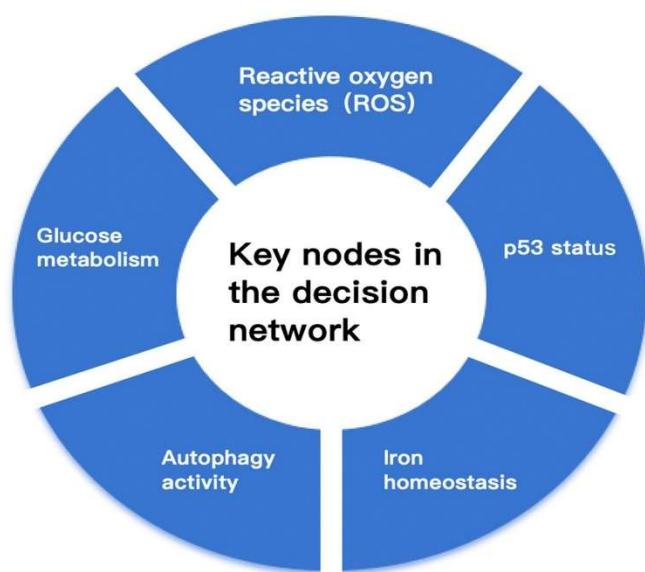


Fig. 2. The key nodes in the decision network.

Iron homeostasis can determine if ferroptosis occurs. PAB impacts on iron homeostasis by increasing transferrin

receptors, ferritinophagy and NCOA4 mediated ferritin degradation [9, 20]. As iron is needed for lipid peroxidation this acts as a permissive step in the network for ferroptosis to occur. This may explain why some cells do not undergo ferroptosis in response to PAB.

These form a network where PAB acts as an upstream regulator and the outcome depends on the context of the cell. This network is shown in Figure 2. These nodes show that PAB acts as an upstream regulator and the outcome depends on the genetic make-up, metabolic state and microenvironment of the cell. This network can help interpret the apparent paradoxes present within the PAB literature. It can also help design experiments to determine the effect of each node on the dominant death mode. This also indicates that the response of PAB as a therapy will depend on how the network is set up within a tumour.

3. FERROPTOSIS AS THE EMERGING CENTER OF GRAVITY

Among the five death modes induced by PAB, ferroptosis has attracted the most attention in recent years. This is due in part to the general growth of the ferroptosis field and in part to the unique mechanisms by which PAB engages this death pathway. Ferroptosis was once regarded as an obscure form of oxidative cell death, but it is now recognized as a major tumor-suppressive mechanism and a promising therapeutic target, particularly for tumors that evade apoptosis. PAB has become a useful chemical probe within this field because it engages the ferroptosis machinery at several distinct points, and the study of PAB has therefore contributed to a broader reappraisal of how lipid peroxidation can be initiated and sustained in cancer cells. This section examines why ferroptosis occupies a privileged position in PAB pharmacology, how PAB engages the ferroptosis network through multiple independent mechanisms, and what the relationship between ferroptosis and the other death modes implies for therapeutic development.

3.1. Why Ferroptosis Is Different

Ferroptosis differs from apoptosis in several important respects. It is iron-dependent and characterized by lipid peroxidation rather than caspase activation [9]. It is morphologically distinct, with shrunken mitochondria and intact plasma membranes [9]. It is regulated by a distinct set of genes, including GPX4, xCT, and NCOA4 [9, 20, 21]. These differences make ferroptosis an attractive target for cancer therapy, particularly for tumors that are resistant to apoptosis. The therapeutic relevance of this distinction is considerable, because many aggressive cancers acquire resistance to apoptosis through loss of p53 function, overexpression of anti-apoptotic Bcl-2 family proteins, or defects in the death receptor pathway, yet they remain sensitive to ferroptosis because the lipid peroxidation machinery is intact and the antioxidant defenses are often

strained by the metabolic demands of rapid proliferation. Ferroptosis therefore represents a death program that is orthogonal to apoptosis, and this orthogonality is the principal reason why ferroptosis inducers are being actively pursued as anticancer agents.

PAB engages ferroptosis through multiple mechanisms that converge on lipid peroxidation. In glioma cells, PAB upregulates transferrin receptor, increasing iron uptake. It also activates Nox4, which generates hydrogen peroxide and lipid peroxides. Simultaneously, PAB inhibits the xCT/GSH pathway through p53-mediated mechanisms, depleting the antioxidant capacity [9]. The combination of increased iron, increased ROS, and decreased GSH creates conditions favorable for lipid peroxidation and ferroptosis. The elegance of this mechanism lies in its convergence: three independent perturbations, each of which alone might be tolerated by the cell, combine to overwhelm the lipid repair capacity of the cell and commit it to ferroptosis. This convergence also explains why PAB-induced ferroptosis is rescued by iron chelation, by lipophilic antioxidants, and by glutathione precursors, because each of these interventions interrupts the cascade at a different point.

In lung adenocarcinoma cells, PAB induces ferritinophagy through NCOA4. This process releases free iron from ferritin stores, increasing the labile iron pool [20]. The excess iron participates in Fenton chemistry, generating hydroxyl radicals that initiate lipid peroxidation. Silencing of NCOA4 attenuates PAB-induced ferroptosis, confirming the importance of this pathway [20]. The involvement of ferritinophagy is mechanistically significant because it distinguishes PAB from inducers that act solely by increasing iron uptake. Ferritinophagy mobilizes iron that is already stored within the cell, and it therefore allows PAB to induce ferroptosis even in cells that do not upregulate transferrin receptor or that have limited extracellular iron availability. This intracellular reservoir of iron may be particularly important in the tumor microenvironment, where iron availability can be variable and where cancer cells often accumulate ferritin as a consequence of altered iron metabolism.

In colon cancer cells, PAB derivatives inhibit GPX4 activity and promote its degradation. GPX4 is the primary enzyme responsible for reducing lipid peroxides. Its inhibition leads to the accumulation of lipid peroxides and ferroptosis. The degradation of GPX4 is mediated by the proteasome, as MG132 reverses the effect [21]. The proteasomal degradation of GPX4 is a particularly effective route to ferroptosis because it removes the principal enzymatic defense against lipid peroxidation rather than merely overwhelming it with substrate. This mechanism also raises the possibility that PAB derivatives could be combined with proteasome inhibitors or with other agents that modulate GPX4 stability, although such combinations would require careful preclinical evaluation because proteasome inhibition can itself influence ferroptosis sensitivity in complex ways.

In non-small cell lung cancer cells, PAB induces ferroptosis through JNK/ERK-mediated upregulation of survivin [22]. This mechanism is distinct from the canonical

ferroptosis pathways. Survivin is an inhibitor of apoptosis protein that also regulates iron metabolism and lipid peroxidation. Its upregulation by PAB contributes to ferroptosis, which is a counterintuitive finding given its anti-apoptotic function [22]. The survivin axis illustrates a broader principle that has emerged from the PAB literature, namely that the molecular determinants of ferroptosis sensitivity are not confined to the canonical xCT-GPX4 axis but include proteins whose primary functions were originally defined in other contexts. This principle is important for biomarker development, because it suggests that the ferroptosis sensitivity of a tumor cannot be inferred solely from GPX4 expression or from the integrity of the xCT transporter. The multiple mechanisms through which PAB engages ferroptosis are summarized in Figure 3, which illustrate the convergence of iron uptake, ferritinophagy, GPX4 degradation, and survivin upregulation on lipid peroxidation.

3.2. PAB as a Multitarget Ferroptosis Inducer

PAB is unusual among ferroptosis inducers in that it engages multiple nodes of the ferroptosis network simultaneously. Erastin inhibits xCT, RSL3 inhibits GPX4, and sorafenib inhibits system xc-. PAB, by contrast, affects iron uptake, iron storage, ROS generation, GSH synthesis, and GPX4 activity [9, 20, 21, 22]. This multitarget engagement may explain why PAB is effective in a broad range of cancer cell types. The multitarget nature of PAB also raises questions about selectivity. Ferroptosis inducers that target a single node may be more selective for cancer cells that are dependent on that node. PAB, by targeting multiple nodes, may induce ferroptosis in a wider range of cells, including normal cells. This is a potential concern for therapeutic development. However, the finding that PAB selectively targets cancer cells over normal cells in some settings suggests that the context dependency of ferroptosis may provide a therapeutic window [1, 22]. The mechanistic basis of this selectivity is not fully understood, but it may reflect the fact that cancer cells often have higher basal iron levels, greater reliance on glutathione synthesis, and higher rates of lipid peroxidation than their normal counterparts, so that the same multitarget engagement produces a greater perturbation in cancer cells than in normal cells.

The multitarget character of PAB also has implications for the interpretation of mechanistic studies. When a single compound engages several nodes of a network, the effect of inhibiting any one node may be partial, because the other nodes continue to drive the process. This is one reason why PAB-induced ferroptosis is often only partially rescued by individual inhibitors, and it complicates the assignment of causality to any single molecular event. The multitarget nature of PAB is therefore both a pharmacological advantage and an experimental challenge, and it argues for the use of systems-level approaches that can capture the combined effect of multiple perturbations rather than relying on single-pathway readouts.

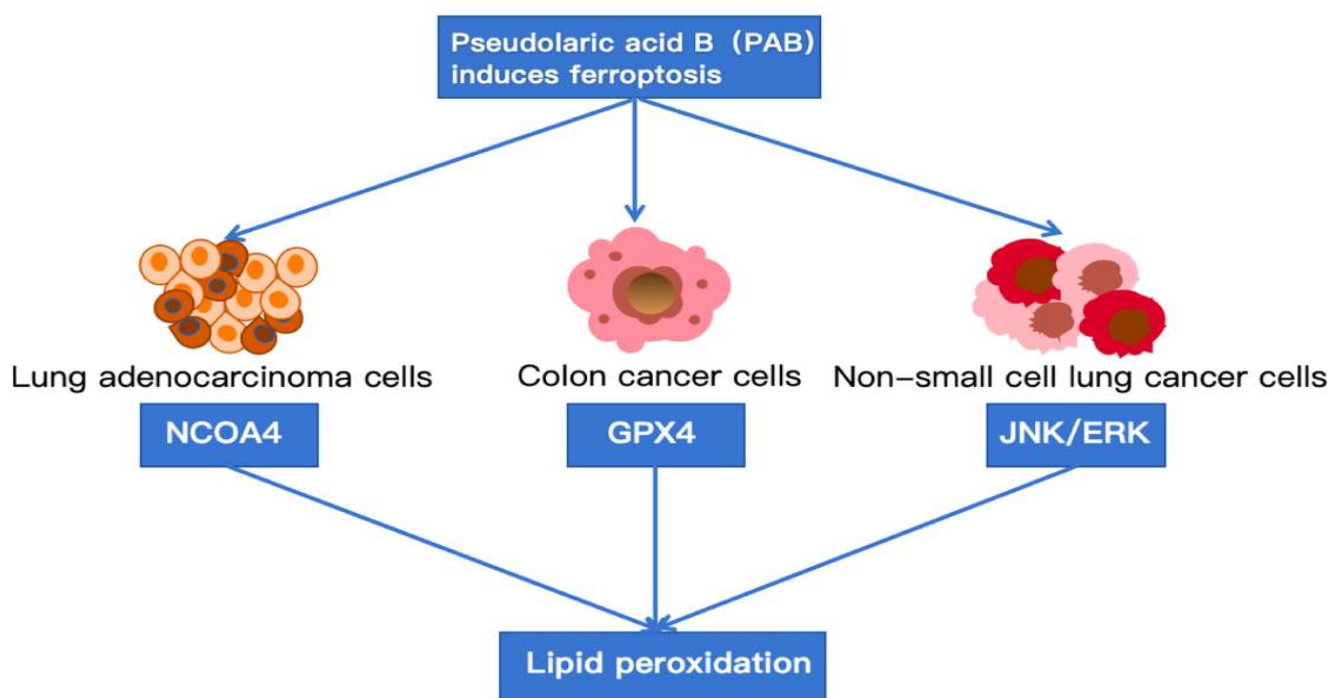


Fig. 3. Pseudolaric acid B induces ferroptosis.

3.3. Ferroptosis and Other Death Modes

The relationship between ferroptosis and other death modes induced by PAB is not well understood. In some settings, ferroptosis may be the primary death mode, with other modes playing secondary roles. In other settings, ferroptosis may be a backup mechanism when apoptosis is blocked. The temporal relationship between different death modes is also unclear. Does PAB induce apoptosis first and ferroptosis later, or do they occur simultaneously? These questions are important for therapeutic development. If ferroptosis is the primary death mode in a particular cancer type, then inhibitors of apoptosis would not block PAB-induced cell death. If ferroptosis is a backup mechanism, then combining PAB with apoptosis inhibitors might enhance its efficacy. Understanding the hierarchy and timing of death modes is essential for rational combination therapy.

The relationship between ferroptosis and autophagy is particularly intricate, because autophagy can either suppress or promote ferroptosis depending on the context. Ferritinophagy, the selective autophagic degradation of ferritin, promotes ferroptosis by releasing iron [20], whereas the autophagic clearance of damaged mitochondria or of lipid peroxidation products can suppress ferroptosis. The same autophagic machinery therefore exerts opposite effects on ferroptosis depending on which cargo is selected, and this duality is likely to be a major source of the context dependence observed in PAB-treated cells. The relationship between ferroptosis and senescence is similarly complex. Senescent cells are metabolically active and often accumulate iron and lipid peroxidation products, which may make them vulnerable to ferroptosis, but the senescence

program also induces antioxidant defenses that can protect against ferroptosis. The balance between these opposing forces may determine whether a cell that enters senescence ultimately survives or undergoes ferroptosis, and this balance is likely to be influenced by the duration of PAB exposure and by the metabolic state of the cell.

These unresolved questions define an important agenda for future research. The development of PAB as a therapeutic agent will require a detailed understanding of which death mode dominates in which tumor context, how the death modes are temporally ordered, and how combinations of PAB with other agents can be designed to exploit the hierarchy among the death modes. The decision network framework introduced in Section 2 provides a conceptual basis for this agenda, and the ferroptosis-centered view presented in this section identifies the specific nodes and pathways that should be prioritized in future studies.

4. FROM MULTI-TARGET TO PRECISION MOLECULE: THE PROTAC APPROACH

PAB is a multi-target natural product that has been shown to bind multiple targets including tubulin, CD147, FLT4, CDK1 and others [4, 30, 33, 34]. Multi-target activity is often viewed as advantageous due to overcoming drug resistance and synergistic effects [1]. However, this leads to concerns of selectivity and off-target toxicities. These factors determine whether PAB should be developed as a conventional cytotoxic agent, where multi-target activity would need to be controlled by formulation and dosing; or whether it can be used as the starting point for rational design

of more selective molecules. Recent efforts have focused on converting PAB from a multi-target cytotoxin into a precision targeted degrader. This section will look at how this has been achieved.

4.1. CD147 Target

CD147 is a transmembrane glycoprotein which is overexpressed in several cancers. It regulates the survival, proliferation, invasiveness and apoptosis of cancer cells [30]. CD147 was identified as a direct target for PAB via the use of chemical proteomics with a PAB derived photoaffinity probe [30]. Further investigations revealed that targeting CD147 with PAB causes apoptosis in acute myeloid leukemia cells. Genetic silencing of CD147 reduced the growth of AML cells and reduced the activity of PAB [31]. Using a photoaffinity probe meant that the target could be discovered within whole cells without any preconceptions of interactions. Genetic silencing also confirmed that CD147 is a mediator to the biological effect of PAB. This method has been used as a template to identify targets for other natural products.

The identification of CD147 as a target led to the development of PAB based proteolysis targeting chimeras (PROTACs). These compounds are bivalent which allows them to bring the E3 ubiquitin ligase into interaction with a target protein causing it to become ubiquitinated and degraded [32]. In 2020 the first PAB based CD147 PROTAC was reported. The model compound 6a caused efficient degradation of CD147 and inhibited melanoma cells in vitro and in vivo [32]. This compound can be used as an innovative anti-cancer agent or as a tool to investigate the dynamic roles of CD147 within cancer. This work has shown that PAB can act as a warhead for the design of PROTACs. A general strategy has been developed to reduce the multi-target activity of PAB to a specific target. CD147 was a good choice of target as it is a transmembrane protein which plays a clear role in cancer. Degradation of CD147 would be expected to cause different effects compared to inhibiting it. This is because PROTACs cause complete degradation of the target protein. This allows disruption of scaffold proteins and protein-protein interactions which wouldn't be possible with traditional inhibitors. CD147 acts as a chaperone for monocarboxylate transporters and a regulator of matrix metalloproteinases so would be expected to cause biological effects based on its presence.

4.2 Other Targets

PAB has also been shown to bind to other targets. FLT4 (VEGFR3) is a receptor tyrosine kinase involved in regulating proliferation and migration in non-small cell lung cancer. PAB shows high affinity binding to FLT4, leading to inactivation of p38 MAPK and PI3K/AKT pathways downstream [33]. CDK1 is a cyclin dependent kinase involved in the G2/M transition. PAB interacts with CDK1 to

reduce both expression and stability, causing G2/M cell cycle arrest in canine mammary tumour cells [34]. GPX4 is a lipid repair enzyme inhibited by PAB derivatives in colon cancer cells [21]. Whilst these are interesting targets for precise targeting, not all are suitable for PROTAC targeting. CD147 is a transmembrane protein, making it suitable for targeting with PROTACs. CDK1 and GPX4 are intracellular targets meaning they may need different targeting strategies [21, 32, 34]. PROTACs have been developed for these targets.

These examples show how the location of the target protein within the cell and how quickly the protein is turned over will influence the choice of E3 ligase, linking geometry and how quickly it will degrade. CDK1 is a cell cycle regulated kinase so degrading this protein would be expected to give similar effects to small molecule inhibitors but may last longer as the protein needs to be resynthesized to restore function. GPX4 is a lipid repair enzyme. Degradation would be expected to cause increased sensitivity to ferroptosis. A PROTAC targeting GPX4 combined with a ferroptosis inducing agent like PAB makes a conceptually attractive method of achieving synthetic lethal effects in ferroptosis sensitive tumours. FLT4 is a receptor tyrosine kinase. Degradation would be expected to give similar effects to kinase inhibition but may be more effective in tumours driven by FLT4 amplification or autocrine VEGF-C signalling. Whilst developing PROTACs for PAB targets is an extension of the CD147 work, they are separate projects.

4.3. Challenges and Opportunities

There are several challenges associated with designing a multi-target natural product into a precise degrader. Due to the number of functional groups within PAB, chemical modifications may alter its overall binding affinity and selectivity [1]. The length and nature of the linker may also affect the degradation efficiency [32]. In addition to this, the choice of E3 ligase may also impact on the target tissue selectivity and toxicity [32]. Overall, whilst these issues are not unique to PAB, they can pose significant challenges due to the multi-target activity within this molecule. As attaching a linker and E3 ligand will alter the surface of the molecule, there could be a disruption in any crucial interactions involved in PAB binding. Therefore, a thorough understanding of the structure activity relationship of PAB will need to be developed to ensure that the correct functional groups are retained to allow targeting of the desired targets.

Overall, this strategy offers many opportunities. As PROTACs will target a specific target for degradation, they may offer improved selectivity and therefore an improved therapeutic index [32]. In addition they can help us understand the role of individual targets in complicated biological systems. The success of PAB PROTAC targeting CD147 demonstrates that this can be applied to other targets within PAB [32]. Overall, this provides a strategy for developing natural products that have previously been difficult to develop due to being multi-target. There are many natural products with therapeutic potential; however they

often suffer from the issue of being multi-target and therefore it can be difficult to determine whether they are effective or toxic. Using PROTACs, this issue can be overcome. This strategy may therefore be applied to many natural products. General strategies for warhead identification, linker optimisation and E3 ligase selection will need to be developed to allow PAB to be used for other natural products. This is likely to be an area of active research for many years. Finally, PROTAC degradation of a target protein is a catalytic process. As PROTACs are not consumed during degradation; they are able to degrade multiple copies of the target. This means that substoichiometric concentrations can be used to create an effect. In addition, the length of action is dictated by the target resynthesis rate rather than the pharmacokinetics of the compound. Whilst this makes PROTACs very appealing as a strategy for targeting, it does introduce new challenges. The interaction between drug concentration, target degradation and overall effect becomes more complex. Therefore, careful pharmacokinetics and pharmacodynamics studies will need to be carried out when designing PAB based PROTACs. Methods for monitoring target degradation will also help. Overall, this strategy holds great promise. However, it shows how much work will need to be carried out between chemists, pharmacologists and clinicians to develop the multi-target natural product into a precise degrader.

5. REMODELING MICROENVIRONMENT

The therapeutic efficacy of PAB is not exclusive to killing tumor cells or pathogens. There is growing evidence that PAB can remodel the disease microenvironment by regulating angiogenesis, immune cell polarization and gut microbiota. While this aspect of their pharmacological profile has received less attention, this can be equally important. Many of the most promising uses of PAB, particularly in inflammatory, metabolic and infectious diseases are likely to be dependent upon their microenvironment remodeling ability. This section will cover the evidence for PAB's ability to remodel the microenvironment by regulating angiogenesis, immune cell polarization and gut microbiota. The implications of these effects for combination therapy will also be discussed.

5.1. Angiogenesis

PAB has multiple mechanisms to inhibit angiogenesis. PAB directly inhibits cell proliferation, migration and tube formation of human umbilical vein endothelial cells [35, 36]. It also decreases VEGF secretion by tumour cells after hypoxia via increased proteasomal degradation of HIF-1 α via PAB [37]. This occurs due to phosphorylation of c-Jun at Ser63/73. Phosphorylation causes the conversion of c-Jun to a non-stabilising transcription factor. This allows PAB to control angiogenesis [38]. This is an interesting concept as it shows how post translational modification can change the

function of a protein by converting it into a different functional form. This also means that PAB is not simply causing toxicity to endothelial cells, but is actually causing the HIF-1 α pathway to be reprogrammed to reduce angiogenesis.

PAB also prevents the anti-apoptotic effects of VEGF on endothelial cells by reducing phosphorylation of KDR/Flk-1 and downstream Akt/ERK signalling [35]. PAB also inhibited angiogenesis and tumour growth in vivo in a mouse model of esophageal squamous cell carcinoma by controlling CD147 [39]. When combined with pemetrexed and Fe(III) in nanoparticles, PAB was shown to inhibit phosphorylation of VEGFR2 and down regulate HIF-1 α . This could be used as a chemotherapeutic anti-angiogenic combination [40]. Interestingly the mechanisms show that PAB can cause inhibition of angiogenesis in many ways. This includes reducing VEGF from tumour cells, and by preventing endothelial cells responding to VEGF. This may be harder for tumours to overcome as they would have to overcome multiple factors to avoid inhibition of angiogenesis. PAB has been shown to reduce tumour growth in vivo in multiple models. This is partly due to PAB having an anti-angiogenic effect [39, 40]. The ability to combine PAB with pemetrexed in nanoparticles is interesting as it shows that PAB can be formulated to increase its tumour targeting. This may reduce systemic toxicity. One concern of PAB being developed as a treatment is that it will have off-target effects. Using nanoparticles to target PAB may help to overcome this.

5.2. Immune Cell Polarization

PAB has been shown to regulate macrophage polarization. In a high salt intake induced hypertrophic mouse left ventricle model PAB was able to reduce M1 macrophage polarization and increase M2 like macrophages [41]. This was accompanied by a decrease in inflammatory cytokine expression and an improvement in cardiac function [41]. In *A. fumigatus* fungal keratitis PAB was shown to reduce corneal inflammation by reducing Mincle/Syk signaling, reducing pro-inflammatory cytokines, and promoting macrophage polarization from M1 to M2 [42]. This demonstrates that PAB consistently promotes M2 polarization across different disease models. PAB acts by suppressing pattern recognition receptors and their downstream signaling pathways. In particular PAB has been shown to reduce Mincle/Syk signaling, a pathway that is activated by fungal ligands and promotes pro-inflammatory cytokine expression. This decreases pro-inflammatory signals, promoting M2 polarization.

PAB also has effects on T cell polarization. PAB inhibits Th1 and Th17 differentiation but increases regulatory T cells via PPAR γ activation and p38MAPK inhibition [43, 44]. Within an in vivo contact hypersensitivity mouse model PAB was applied topically and was able to reduce ear oedema and inflammatory infiltration by regulating Th1/Th17/Treg polarization [45]. In T cell mediated immune responses PAB inhibits p38MAPK signaling and activates PPAR γ leading to

reduced TNF- α production [43]. Activation of PPAR γ is key in determining the anti-inflammatory effects of PAB. PPAR γ is a known regulator of anti-inflammatory responses therefore its activation leads to reduced inflammatory responses. PAB also inhibits p38MAPK signaling that is known to be involved in inflammatory cytokine production in both innate and adaptive immune cells. PAB has the ability to reduce Th1 and Th17 responses whilst increasing regulatory T cells. This is important within autoimmune and inflammatory diseases as often there is a bias towards effector T cells.

In terms of cancer, PAB has immunomodulatory effects. M2 macrophages and regulatory T cells within the tumor microenvironment are typically involved in immunosuppression and poor patient outcome. Whereas M1 macrophages and effector T cells are involved in antitumor immunity. Therefore, in an inflammatory setting where PAB would increase M2 polarization this could be seen as adverse for cancer. However, this effect is dependent upon the context of the macrophage polarization. In the tumor microenvironment PAB derivatives have been reported to cause reprogramming of tumor-associated macrophages from an M2 to M1 phenotype [48]. This fits with the decision network mentioned in section 2 as the outcome depends on the context of the cell and microenvironment. Therefore, PAB may be beneficial in cancer due to its ability to reverse immunosuppressive polarization.

5.3. Gut Microbiota

PAB has been shown to have beneficial effects on gut microbiota in a murine model of metabolic dysfunction associated fatty liver disease (MAFLD). It was found to ameliorate symptoms of MAFLD whilst repairing the dysbiosis of intestinal microbiota [46]. The abundance of harmful bacteria such as *Faecalibacterium*, *Allobaculum*, *Ileibacterium* and *Dubosiella* were reduced following administration of PAB [46]. Fecal microbiota transplantation confirmed that this effect was due to PAB influencing the community structure of the microbiota [46]. A major advantage of this study was that fecal microbiota transplantation was used to provide evidence of causation that the microbiota is a mediator for some of the therapeutic effects of PAB. This would not have been possible without causal evidence to suggest that changes to the microbiota are due to the administration of PAB rather than confounders.

This was due to PAB increasing the abundance of tryptophan metabolism. By increasing the abundance of the microbial metabolite cinnabaric acid which binds to and activates the aryl hydrocarbon receptor (AhR) [46]. Activation of the AhR gene leads to decreased expression of lipogenesis genes in the liver via the IL-22/JAK1/STAT3 pathway [46]. This has revealed that PAB can protect against MAFLD via modulating the intestinal microbiota, its metabolites and the host [46]. This is an important finding as it has identified a specific metabolite produced by the microbiota that is responsible for mediating PAB's effects.

This has provided a molecular link between the gut microbiota and the host liver and has identified a potential biomarker. The involvement of AhR is consistent with PAB having an overall immunomodulatory effect as it is a key regulator of immunity in the intestine and interactions between the microbiota and host.

PAB demonstrates increased therapeutic efficacy in comparison to traditional antibiotics in a murine model of vaginitis caused by *Candida albicans*. It restored normal tissue architecture, reduced inflammation and increased the abundance of beneficial vaginal microbiota [47]. PAB also showed direct anti-fungal effects by inhibiting biofilm formation and decreasing ergosterol biosynthesis [47]. This is a highly attractive treatment option for mucosal infections as it can target the infection directly but also modulate the microbiota. The ability to inhibit biofilm formation is an important property as they are a major cause of resistance to traditional anti-fungal agents.

5.4. Implications for Therapy

PAB derivatives have important implications for therapy due to their ability to reprogram the disease microenvironment. In cancer, PAB derivatives could improve anti-tumour immunity by reprogramming tumour-associated macrophages into an M1 phenotype from an M2 phenotype [48]. They could also increase drug delivery through the normalization of tumour vasculature. In inflammatory diseases, PAB could reduce tissue damage by repolarizing macrophages and controlling T cell populations [43, 45]. In metabolic diseases PAB could aid liver function via alterations in gut microbiota [46]. This suggests that PAB could be a valuable addition when used alongside other therapies which aim to alter the microenvironment. For example, PAB could be administered alongside immune checkpoint inhibitors to increase anti-tumor immunity. Or alongside antibiotics to help restore microbial homeostasis.

Using PAB alongside immune checkpoint inhibitors is an appealing strategy as they can act synergistically. Immune checkpoint inhibitors reduce the inhibition of effector T cells whereas PAB could increase macrophage polarization and vascular normalization. Using PAB alongside antibiotics or other modulators of the microbiota would also be appealing in infectious and metabolic diseases as they would allow both the cause of the disease to be targeted and the dysbiotic community. Although these strategies would require careful pre-clinical investigation to ensure optimal dosing, timing and order; using PAB alongside other therapies demonstrates potential for the therapeutic development of PAB. Overall, the effect PAB has on the disease microenvironment suggests that clinical development should not be limited to oncology as its ability to modulate inflammation, metabolism and microbiota could be exploited across a range of diseases.

6. THREE KEY QUESTIONS TO ADDRESS IN FUTURE WORK

The field of PAB research has matured to the point that it is now necessary to look beyond descriptive studies. To advance this field we need to understand the mechanisms of PAB and how they can be used therapeutically. There are three key questions that should be addressed in future PAB work. While the answers to these questions will inform each other they can also be considered separately. Firstly we need to know how to predict the mode of cell death induced by PAB. Secondly we need to know if we should focus on developing PAB to induce ferroptosis. Thirdly we need to know if the PROTAC approach can be applied to other natural products. By answering these questions we can turn PAB from an unpredictable but interesting natural product into a rational therapeutic.

6.1. How can we predict PAB-induced cell death pathways?

A challenge associated with the development of PABs is that they will induce different cell death pathways depending on the cellular context (apoptosis, ferroptosis, autophagy, senescence, mitotic catastrophe) [7-9,25]. This variability makes it difficult to identify which patient will respond to treatment, and could hinder clinical trial designs. Clinical trials may fail to show benefits if they are using unselected populations, where only those within a biomarker-defined group would response. The solution to this issue is not to prevent the dependence on cellular context, but to understand how these differences occur and make predictions based on this knowledge.

To predict the pathway of cell death induced by PAB we need to identify biomarkers. Several markers have been identified including p53, GPX4, glucose utilisation and autophagy. p53 status determine whether senescence or apoptosis occurs [7, 25]. GPX4 levels will determine susceptibility to ferroptosis [21]. Glucose utilisation determines whether senescence occurs or apoptosis [25]. Autophagy levels determine if the cells are protected by autophagy or if it will cause death [10, 11]. These biomarkers could be used to select patients for clinical trials. For example patients with wild-type p53 tumours may have more senescence whilst patients with p53-null tumours may have more apoptosis [7, 25]. Patients with high levels of GPX4 may be resistant to ferroptosis whilst low levels may be susceptible [21].

Due to death pathway being dependent on the whole decision network, rather than individual nodes, the panel of predictive biomarkers will have a higher predictive value compared to individual markers. This suggests that multivariate techniques such as machine learning should be used to develop a panel of markers. They should also be validated in prospective clinical samples rather than retrospective cell line studies due to the possible changes in the relationships caused by the microenvironment and genetic heterogeneity of human tumours. The decision network (section 2) provides a good framework to select candidates. The important nodes

including ROS, p53, glucose, autophagy and iron homeostasis were identified as the most promising biomarkers to develop a panel.

6.2. Should Ferroptosis Be the Primary Focus of PAB Development?

Ferroptosis has become a key mechanism of cell death in PAB [9, 20-22]. PAB's ability to target multiple points within the ferroptosis pathway makes it unique compared to other ferroptosis inducing agents. Whether ferroptosis should be the main target of PAB development is important to consider, as it will influence the cancer types targeted, the biomarkers developed and the combination therapies explored.

There are many reasons why ferroptosis should be the main target. Firstly, PAB is very effective at killing apoptosis resistant cancer cells [9]. The multi-targeting of multiple points within the ferroptosis pathway may result in overcoming these resistance mechanisms [9, 20-22]. Secondly, ferroptosis is immunogenic so PAB may be effective as a combination therapy. This is an exciting property to have as it indicates that PAB may have off target effects, and therefore help control distant metastases. Other ferroptosis inducing agents have been seen to have an abscopal effect, therefore there is a rationale for using PAB in combination with immune checkpoint inhibitors.

However, there are also reasons why ferroptosis may not be the best target. PAB induces multiple modes of cell death [7, 8, 25], therefore in certain cancer types apoptosis or senescence may be more important. Whilst targeting multiple points within the ferroptosis pathway, there may be increased toxicity to normal cells [1]. The therapeutic window may be small. This is an important point to consider. Since PAB targets multiple points in the ferroptosis pathway, it may cause ferroptosis in any normal cells which still have functional ferroptosis machinery. This could therefore limit the maximum dose tolerated. The narrow therapeutic window is a concern for a drug that will be administered systemically, so targeting PAB specifically to tumour cells or using prodrug forms may be beneficial.

A middle ground may be to target PAB to specific indications where we know that ferroptosis is important. For example, PAB could be targeted at glioblastoma as we know it is effective [9]. Alternatively, cancers with low GPX4 expression could be targeted as they will be more susceptible to ferroptosis [21]. This will ensure maximum therapeutic window in each cancer type and the biomarker panel can be personalised to the most relevant cell death mechanism.

6.3. Can the PROTAC Strategy Be Extended to Other Natural Products?

The success of the PAB CD147 PROTAC indicates that this strategy can be applied to other natural products [32]. Many natural products have multiple targets and are not selective. To overcome this PROTACs can be synthesised to

specifically degrade the target without any off-target effects [32]. This is an interesting strategy to apply as many natural products have poor selectivity due to them having multiple targets.

This strategy can be difficult to apply to natural products as they can have complex structures and multiple functionality [1]. The binding characteristics may change upon modification to form PROTACs [1]. The length and composition of the linker can also affect how efficient degradation will be [32]. Selecting the correct E3 ligase can affect how specific the degradation will be to different tissues and any potential toxicity [32]. However, most natural products do not have well defined targets. Therefore a warhead must first be identified that has high affinity and selectivity for the target. A chemical proteomics approach like that used to identify CD147 as a PAB target can be used. This will help to speed up the creation of PROTACs for other natural products.

Overall this strategy looks promising. It allows natural products to go from being non-specific cytotoxins to becoming highly targeted degraders [32]. It can also provide an insight into the role of individual targets within complicated biological systems. This is an actively researched area with many researchers working on creating PROTACs for other natural products including curcumin, resveratrol and berberine. To be successful general methods for identification of warheads, optimisation of linkers and selection of E3 ligases will need to be developed. This will require collaboration between chemists, biologists and pharmacologists. The PAB CD147 PROTAC shows this can be successful, meaning it could be seen as a model PROTAC.

7. CONCLUSIONS

PAB is a natural compound, which can induce multiple death pathways in cells including apoptosis, ferroptosis, autophagy, senescence, and mitotic catastrophe [7-11, 25, 26]. Therefore, it is difficult to develop PAB as a conventional drug due to the cell context dependency. However, the unique cell context dependency of PAB can also be exploited to target specific cancer types according to the genetic and metabolic profiles of the cancer. So far, a lot of descriptive studies have been published [1-3]. Therefore, it is critical to develop a holistic picture to rationalize the data. Here, we propose a cell fate decision network to provide an overview of how cell fate is determined. This network identifies the key nodes, predicts potential biomarkers, and emphasizes the emerging ferroptosis hub. We also discuss how PAB, a multi-target natural compound, can be converted to a targeted degrader. Future studies are needed to validate the network, identify biomarkers, and develop PAB-based PROTACs. In clinical trials, patient stratification according to p53 status, GPX4 expression and metabolic profiles should be performed. Moreover, combination therapies should take advantage of the remodelling properties of PAB. This may allow PAB to fulfill its potential as a therapeutic for cancer, fungal infection and metabolic disease.

DECLARATIONS

Ethical Approval

We affirm that this manuscript is an original work, has not been previously published, and is not currently under consideration for publication in any other journal or conference proceedings. All authors have reviewed and approved the manuscript, and the order of authorship has been mutually agreed upon.

Availability of data and material

All of the data obtained or analyzed during this study is included in the report that was submitted.

Conflicts of Interest

The authors declare that there is no conflict of interests.

Authors' contributions

All authors contributed equally to this work.

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