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REVIEW ARTICLE

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Mitochondria: Endosymbiont bacteria DNA sequence as a target against cancer

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Abstract

As the energy factory for the cell, the mitochondrion, through its role of adenosine triphosphate production by oxidative phosphorylation, can be regarded as the guardian of well regulated cellular metabolism; the integrity of mitochondrial functions, however, is particularly vulnerable in cancer due to the lack of superstructures such as histone and lamina folds to protect the mitochondrial genome from unintended exposure, which consequently elevates risks of mutation. In cancer, mechanisms responsible for enforcing quality control surveillance for identifying and eliminating defective mitochondria are often poorly regulated, and certain uneliminated mitochondrial DNA (mtDNA) mutations and polymorphisms can be advantageous for the proliferation, progression, and metastasis of tumor cells. Such pathogenic mtDNA aberrations are likely to increase and occasionally be homoplasmic in cancer cells and, intriguingly, in normal cells in the proximity of tumor microenvironments as well. Distinct characteristics of these abnormalities in mtDNA may provide a new path for cancer therapy. Here we discuss a promising novel therapeutic strategy, using the sequence-specific properties of pyrrole-imidazole polyamide-triphenylphosphonium conjugates, against cancer for clearing abnormal mtDNA by reactivating mitochondrial quality control surveillance.

KEYWORDS

age-related disorder, anticancer therapy, apoptosis, autophagy, Bcl family, exocytosis, mitochondria, mitochondrial disease, mitochondrial quality control, mitophagy, mtDNA, mutation, polymorphism, pyrrole-imidazole polyamide, reactive oxygen species, senescence, triphenylphosphonium

1 | INTRODUCTION

Mitochondrial dysfunctions in cancer are heterogeneous and complex, due to the wide range of mutations in the diploid nuclear and haploid mitochondrial DNA (mtDNA), or manifested in the high volume of mtDNA copies found in individual cells. Despite the diminutive size of the mitochondrial genome at 16.5 kbp, the amount of frequent mutations found in the 37 genes encoded within often exceed and affect the preservation of mitochondrial structure and function to a greater extent compared with the more than 1500 related genes in the nuclear genome.[1] While a properly functioning system of mitochondrial quality control (MQC) can identify and

excise these mutations, the infrastructure responsible for mitochondrial regulation is often poorly regulated and crumbling in a large number of cancers.[2] As acquired pathogenic mutations in cancer are occasionally homoplasmic or near-homoplasmic,[3,4] the mere presence of such mutations can have irrepressible consequences in metabolism; similar to the Warburg effect, cancer cells with these homoplasmic mitochondrial mutations can undergo metabolic reprograming, causing oxidative phosphorylation of ATP and harmful cellular damage as a consequence of reactive oxygen species (ROS) production[5] and oncometabolites such as fumarate, succinate, and 2-hydroxyglutarate.[6] Abnormal mitochondria often trigger inflammatory or cell death processes in a similar manner as seen in bacterial infection,[7] possibly because of the endosymbiotic theory that mitochondria are endosymbiotic organelles originated from ancestral alphaproteobacterium.[8] In both intrinsically and extrinsically regulated cell death, for instance apoptosis, necrosis, pyroptosis and ferroptosis (a process intimately connected to both positive and negative machinery for cancer initiation, promotion and propagation[9]), as well as aging processes such as cellular senescence,[6] the involvement of mitochondria further highlights the necessity of functioning MQC. Particularly, aging is well associated with cancer susceptibility risks and the extent of mtDNA mutations, a process by which induces cellular senescence and the associated secretory phenotypes (SASPs) for triggering pro-inflammatory and pro-oncogenic secretion.[10] Abnormal mitochondria accumulation is a critical event in cancer development and progression[3,4,11-13] and the need to identify, isolate, and excise these mtDNA aberrations is critical for cancer therapy.

When it comes to devising a solution for a difficult problem, a simple and straight-forward solution is perhaps the most preferable; attenuating the rise of mitochondrial genome aberrations, the ability to interact directly with mtDNA via locus-specific manipulation is also perhaps the most logical solution. Pyrrole-imidazole (PI) polyamides, with their proven track record in nuclear genome binding in a sequence-specific manner, therefore show great promise for mtDNA-based applications. Chemical conjugation of PI polyamides with various moieties, often referred to as PI polyamide-drug conjugates (PDC), can expand their functional repertoire beyond simple minor groove base-specific binding; these PDCs are capable of genomic manipulation down to the precision of a single nucleotide difference in vitro and in vivo.[14] Expanding upon this strategy of "genome instigators," we and others around the world have designed and reported various PDCs that can target specific double-stranded B-form DNA sequences in the nuclear and mitochondrial genomes, as well as genomes of parasitic microbes to provide means of therapy,[15-24] as well as cancer diagnostic applications in the form of target enrichment from bodily fluid specimens.[25]

Here we discuss some of the more recent developments in cancer mtDNA homing, and the reactivation MQC surveillance of mutant mtDNA as means to trigger homoplasmic mtDNA mutants removal in cancer and the tumor microenvironment (TME), via the phenomenon of ROS overproduction and the subsequent cell death (Figure 1). The approach of initiating mutant mtDNA clearance by mitophagy may be extended to formulate an agnostic therapy for cancers with homoplasmic mtDNA mutation, and be extended to other diseases such as mitochondrial disorders, diabetes, deafness, neurodegenerative diseases, and age-related disorders by targeting the respective driver as well as passenger mtDNA loci.

2 | MITOCHONDRIA AND CANCER

At 16 569 bp, the circular human mitochondrial genome is a double-stranded DNA encoding 13 subunits of oxidative phosphorylation (OXPHOS) enzyme complexes I, III, IV, and V as well as 22 tRNAs and 2 rRNAs.[26] Perhaps due to its size, copy number, restricted repair mechanisms, and the lack of protective nucleosomal structures, mutations in mtDNA occur at a frequency approximately 10-fold higher than their nuclear counterparts regardless of species.[27] Some diseases often exhibit signs of reduced ATP production, ROS elevation, induction of mitochondrial membrane permeability transition (MPT), and/or initiation of mitophagy, all of which subsequently cause mitochondria-related inflammation, senescence, or cell death, with extracellular release of pro-oncogenic/inflammation factors as well as pathogenic mitochondria.[10,28-30] mtDNA mutations are responsible for not only mitochondrial diseases, also known as pediatric metabolic disorders, but also aging and lifestyle-related diseases such as diabetes, cancer, and neurodegenerative disorders. While maternally inherited mitochondrial disease, or for that matter any haplotype with respiratory chain complex deficiency without ROS increase, has not been associated with cancer incidence directly, decreases in respiratory activity, and increased ROS production, both of which can be attributed to the mitochondrion, do infer with the organelle's involvement in cancer progression leading to hypoxia, disruption in metabolic homeostasis, as well as DNA and protein damage.[7,28]

We previously investigated the mitochondrion's contribution to metastatic potential in cancer, and found that nuclear and cytoplasmic exchange of highly metastatic mitochondria with the G13997A mutation and low-metastatic cancers with wild-type mtDNA could lead to the displacement of mitochondria in low-metastatic cells with those from highly metastatic cells, to confer increased metastatic potential. Conversely, we observed no signs of metastasis in the highly metastatic cells that incorporated mitochondria from low-metastasis cells.[11] We also generated 2 different mice lines, one harboring an mtDNA mutation causing decreased respiratory chain complex activity and the other with an mtDNA mutation related to decreased respiratory function as well as ROS overproduction in germ cells. The mice with ROS-overproducing mutant mitochondria developed diabetes and lymphoma, along with hyperlactic acidosis; conversely, mice with only reduced OXPHOS capacity showed hyperlactic acidosis, but were alternatively diabetes and lymphoma free. Intriguingly, administration of the antioxidant *N*-acetyl cysteine (NAC) suppressed the onset of diabetes and lymphoma.[12] Mitochondrial mutations were indeed very frequent and often pathogenic in colorectal cancers and non-small-cell lung carcinomas

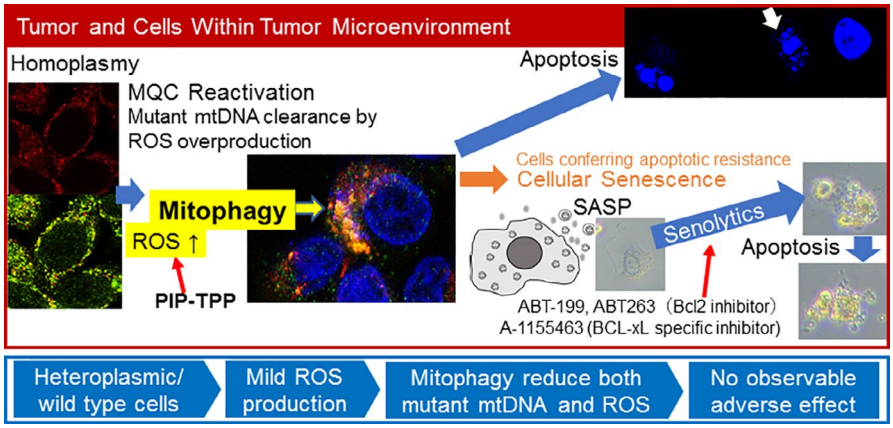

FIGURE 1 Anticancer and antisenescence mechanisms targeting mutant mitochondria. Cancer cells exhibit diminished exclusion inhibition and retention of dysfunctional mitochondria as a consequence of misregulation in mitochondrial quality control (MQC) systems and the high mutation rate of mtDNA. Once accumulation of pathogenic mtDNA mutation is initiated, the gradually increased number of pathogenic haploid genome can either to induce cell cycle arrest or lead to a plastic cell growth state consisting of primarily homoplasmic mutations. PIP-TPP designed to recognize mtDNA mutation sequence are able to locate in mitochondria and should bind mutant mtDNA; the disruption can induce mitophagy through MQC surveillance reactivation. Apoptosis induced as a consequence of ROS overexpressing mitophagy occurs in homoplasmic or near-homoplasmic cells, while wild-type or heteroplasmic cells, such as normal cells, can survive after PIP-TPP exposure. While cell death-resistant cancer cells may survive, cellular senescence soon develops. These resistant cells, which often express anti-apoptotic Bcl family genes, can be overcome using Combination (CMBO) with senolytics like pan-Bcl inhibitor or Bcl-xL specific inhibitor, all of which will ultimately induce cell death

(NSCLCs), especially in metastatic cancers; as these pathogenic mutations were homoplasmic, the phenomenon of mitochondrial displacement meant that the tumor and the nearby non-cancerous cells in these TME most certainly harbored these mitochondrial mutations[13]; somehow, preferential mtDNA mutations must have been selected during the bottleneck process in cancer and TME propagation,[3,4,13] and these mitochondria escaped MQC surveillance and continued to proliferate in the TME via mechanisms such as gene fission or lateral gene transfer.

Mitochondria hold a central and multifunctional role in oncogenesis; targeting the various mitochondrial-related biological functions can provide therapeutic opportunities that span not only biochemical metabolism but also apoptosis, hypoxia, and the immune response.[2,6,10,31] Recently, some small and medium molecular weight drug candidates, as well as biologics and genome editing methods, for instance RNA interference, antisense, cyclic peptides, TALEN, CRISPR technology, antibodies and antibody-like biologics,[28,32-36] have made significant strides in targeting mitochondrial mutations or related proteins targets. At the time of writing, however, there are currently no clinical approaches that seek to target mutant-specific mitochondria for cancer treatment, and further study is highly warranted in this area.

3 | ANTIOXIDATIVE DEFENSE AND CANCER

Antioxidative defense strategies are invoked by cancer cells to maintain ROS levels in tolerable cell survival level.[37] ROS is an intrinsic mutator mainly generated from mitochondria and contributes to the control of cell proliferation and differentiation at moderate level. Tight regulation of ROS levels is crucial for cellular life even in cancerous cells.[38] Cancer cells often have dysfunctional mitochondria and increased mitochondrial ROS (mitROS),[37] which is strictly regulated by oxidative defense mechanisms[7] similar to cancer caretaker genes.[39] Among these, mitophagy is the selective removal and degradation of damaged mitochondria by autophagy and induced by the precursors of the peripheral division with MPT, ROS overproduction, higher Ca^{2+}, and often non-replicating mtDNA inclusion.[7,30] Mitophagy are able to minimize harmful ROS produced by peripheral division. For instance, in Cos-7 cells transformed from normal African green monkey kidney, ROS producing divisions occur at a similar frequency to ROS non-producers. This phenomenon is observed to a lesser extent in mouse neonatal cardiomyocyte cells, the defense against ROS by mitophagy appears to be general in transformed cells and species independent.[30]

Oxidative stress response genes are regulated by a *cis*-acting enhancer sequence of the antioxidant response element (ARE) that enhances expression of proteins responsible for controlling the cellular redox status and defense against oxidative damage.[40] Cancers are likely to have expressional addictions in genes participating in the ARE pathway, for example NRF2, and these genes are often considered potential therapeutic targets.[41] The mitochondrial uncoupling protein (UCP) family are a family of mitochondrial anion carrier proteins capable of alleviating mitROS production.[42] For instance, UCP2 upregulation is frequent in some cancers and can be consistently observed in multiple cancer cell lines promoting a highly glycolytic phenotype, inhibition of ROS accumulation, and apoptosis-resistance after exposure to chemotherapeutic agents.[43] UCP2 is anchored in

the inner mitochondrial membrane (IMM) and competes for protons in the electron gradient for ATP synthesis; the protein maintains energy balance during phosphorylating conditions, and indirectly protects mitochondria against ROS by diminishing the reduced state of the respiratory chain.[44]

In general, ROS scavenging systems and oxidation–reduction (redox) shifts are well observed events in cancer development. Cellular overflow of oxidizing substances of radical ROS, non-radical ROS, and reactive nitrogen species in tumors are also scavenged by redox such as glutathione and superoxide dismutase (SOD).[45] Glutathione is the most abundant low-molecular-weight thiol, it is the major redox pool in most cells and the target for antioxidant therapy,[46] while SODs are frequently overexpressed in tumors and, therefore, are considered to be anticancer drug targets.[37,47,48] As the mitochondrial glutathione concentration is similar to that of the cytosol, the role of redox defense in the mitochondria by the GSH regulatory system can be expected to perform on a similar level as the rest of the cell[49]. However, when taking into consideration the presence of a steep electrochemical gradient across the mitochondrial membrane needed for energy production, it becomes clear that any disruption to redox homeostasis can shift cells into a diseased state, especially when an organelle as critical as the mitochondrion is stressed.

The disruption of oxidative defense mechanisms in cancer can provide a direct route for cancer therapy via a synthetic lethality known as poly ADP ribose polymerase (PARP) inhibitors which facilitate cancer cell death in those with excessive error-prone phenotypes due to double functional defects of DNA double-stranded break repairs of nonhomologous end joining and single-strand annealing.[50] Weakened oxidative defense mechanisms under mitROS overproduction in cancer are barely controlled situations similar to BRCAness, and oxidative defense mechanisms are therefore considered to be great cancer synthetic lethality targets without affecting non-cancerous cells.[37,48]

4 | MITOCHONDRIAL QUALITY CONTROL SURVEILLANCE

The accumulation of dysfunctional mitochondria is often an outcome of programmed/unfavorable cellular consequences of some pathological changes in cancers as well as aging and neurodegeneration. Due to the high mutation rate of mtDNA, dysfunctional mitochondria are always present in cells, but are promptly cleared by autophagocytosis or exocytotic excretion prior to accumulation to ensure cellular homeostasis. At the organelle level, mitochondria undergo self-renewal by non-selective autophagic and selective mitophagic processes; abnormal mitochondria are detected and subject to peripheral fission to generate fragmented mitochondria that can be cleared by autophagic processes such as mitophagy[30] or by excretion by exocytosis via subsequent neutrophil and macrophage phagocytosis. Although the mechanism behind MQC remains unclear, possible molecular mechanisms

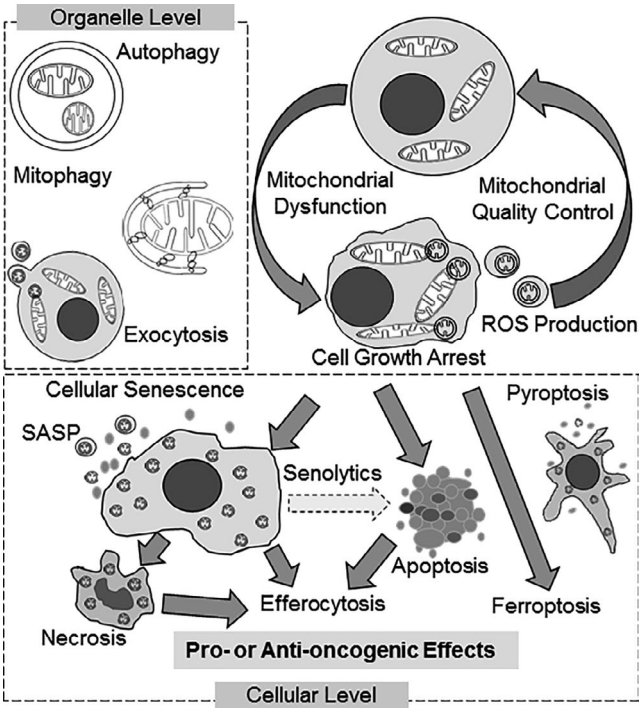

FIGURE 2 Mitochondrial quality control. Dysfunctional mitochondria are always present in cells due to the high mutation rate of mtDNA, but are typically removed by autophagocytosis or excreted by exocytosis to ensure cellular homeostasis. Mitochondria are self-renewed by non-selective autophagy and selective mitophagy or excretion process of exocytosis, with subsequent neutrophil and macrophage phagocytosis. Accumulated mutant mtDNAs in cell will induce cell cycle arrest, but are also removed via apoptosis, necrosis, ferroptosis, pyroptosis, and efferocytosis or even cellular senescence

have been discussed in the current literature.[7,51,52] At the cellular level, accumulated mutant mtDNAs are also removed, as the whole cell undergoes cell death via apoptosis, necrosis, ferroptosis, pyroptosis, or efferocytosis[53] (Figure 2). Nevertheless, some physiological mutations can evade MQC and are retained in cancer cells, perhaps by chance or by oncological processes yet to be elucidated. Mitophagy induces the perturbation of mitochondrial respiration leading to MPT induction and ROS production; promotion of oxidative defenses including mitophagy cannot dissipate enhanced MPT-mediated death or cellular senescence.[7] Cell death and senescence generally promote anti-oncogenesis. However, at times, these events also work as pro-oncogenic secretary phenotypes through damage-associated molecular patterns, pathogen-associated molecular patterns, and SASP. Abnormal mitochondria are routinely released outside the cell and transferred by tunneling nanotube transfer (TNT), or indirectly by exocytosis via migrasomes, exosomes, mitovesicles, or other extracellular vesicles (EVs)[29,52,53] (Figure 3). Although most EVs are either excreted or subjected to endocytotic events by phagocytic cells such as macrophage and neutrophils, a proportion can be transferred into neighboring cells. This may partially explain why cancer cells and surrounding cells in the TME develop a tendency to acquire

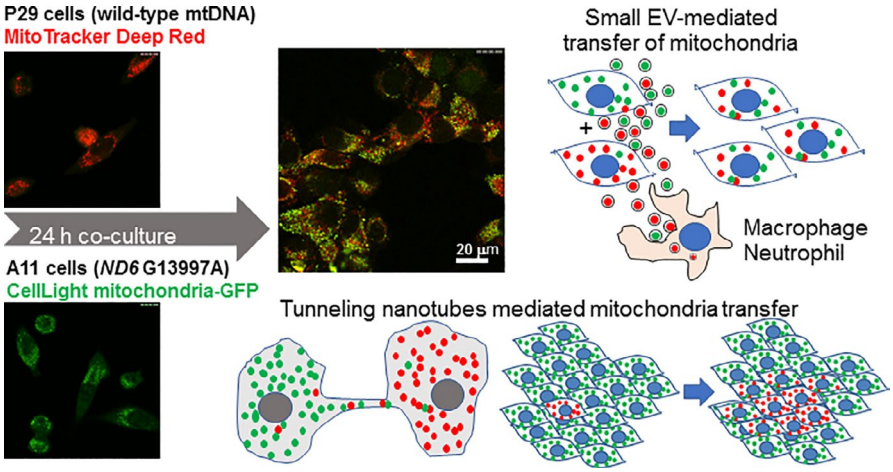

FIGURE 3 Intercellular mitochondrial transfer in cancer cells. When co-cultured with 2 cancer cells, 2 types of mitochondria from each cell are mixed. Mutated mitochondria are released outside of the cell and transferred to the neighboring cells by exocytosis using migrasomes, exosomes, mitovesicles, or the other extracellular vesicles (EVs) or directly by TNT. The pathogenic mtDNA haplotype is expanded and spread to neighboring cancer and TME cells. This could promote cancer progression and induce pro-oncogenic tumor microenvironment

characteristically homoplasmicity, and contain nearly identical mtDNA mutations, subsequently promoting a pro-oncogenic microenvironment.

5 | PYRROLE-IMIDAZOLE POLYAMIDE: SYNTHETIC MINOR GROOVE BINDERS MIMICKING ANTIBIOTICS

Minor groove binders (MGB) of microbial origins are naturally occurring antibiotics, with mitomycin and distamycin being particularly of note due to their ability to recognize GC and AT pairings, respectively. Expanding upon these ideas, Dervan and Sugiyama in recent years have pioneered developments in synthetic MGBs that recognize specific DNA sequences. They discovered that distamycin containing 3 rings of *N*-methylpyrrole that recognizes B-DNA in a binding configuration of antiparallel dimers in the minor groove.[54,55] Dervan elegantly developed sequence-specific *N*-methylpyrrole (Py) and *N*-methylimidazole (Im)-containing hairpin polyamides to recognize and bind DNA minor grooves through hydrogen bonding interactions in a base-dependent fashion, in which Py could tightly bind A, T, C via a single hydrogen bond and Im similarly recognized G via 2 hydrogen bonds to allow Py-Py pairing to bind preferentially A:T or T:A and Im-Py with G:C pairings.[15] These "PI" polyamides can be modified by conventional synthesis techniques found in polyketide and amino acid chemistry, and by the introduction of substituents into various positions (R1–R5 in Figure 4A) can improve their DNA recognition or outfit the polyamide with additional functionalities, for instance delivering a functional small molecule conjugated to the polyamide, typically at the N- or C-terminus, to genomic DNA in the cell. We and others have successfully synthesized various PDC that target genomic regions in the normal/disease genome, and subsequently evaluated those conjugates, both in vitro and in vivo, to confirm their anti-disease efficacy as well as genetic or epigenetic modification to the target genes[17,23,56,57] (Figure 4B).

PDCs designed as anticancer agents frequently displayed the ability to alter the genetic or epigenetic state of their genomic targets, and mouse models of various human cancers also confirmed the anticancer efficacy of PDCs, simultaneously showing few adverse events.[16,18-21,58-62] Intriguingly, pharmacokinetic studies also suggested that PI polyamide conjugates possessed enhanced permeability and retention (EPR)-like effects,[63] an additional advantage for PDCs to localize preferentially in tumors and the surrounding environments, as expected of well performing cancer therapeutics.[64] Several modifications to a candidate PDC could also improve its intracellular localization, for instance homing toward the mitochondria.[22,59] Druggability, synthesizability, and modifiability of PDC, coupled with the capability of targeting disease genomes at specifically affected lesions/locations,[15] allowed to develop a new strategy for cancer treatment against a variety of unfavorable/rare tumors.[16,18,20,21,60-62,65] PDCs may also be used as companion diagnostic tools for liquid biopsy to enrich and identify stealth mutations[25] and as a fluorescent probe to detect chromosomal region.[66]

6 | PI POLYAMIDES CAN TARGET THE MITOCHONDRIAL GENOME

It had been initially proposed that a PI polyamide targeting mutant mtDNA could create an obstacle for replication; it was shown later that hairpin-type PI polyamides targeting the 3243G MELAS mutation could marginally reduce the expression of both normal and mutant mtDNA in heteroplasmic cells. This result could be explained by the finding that mtDNA-bound PI polyamide transiently

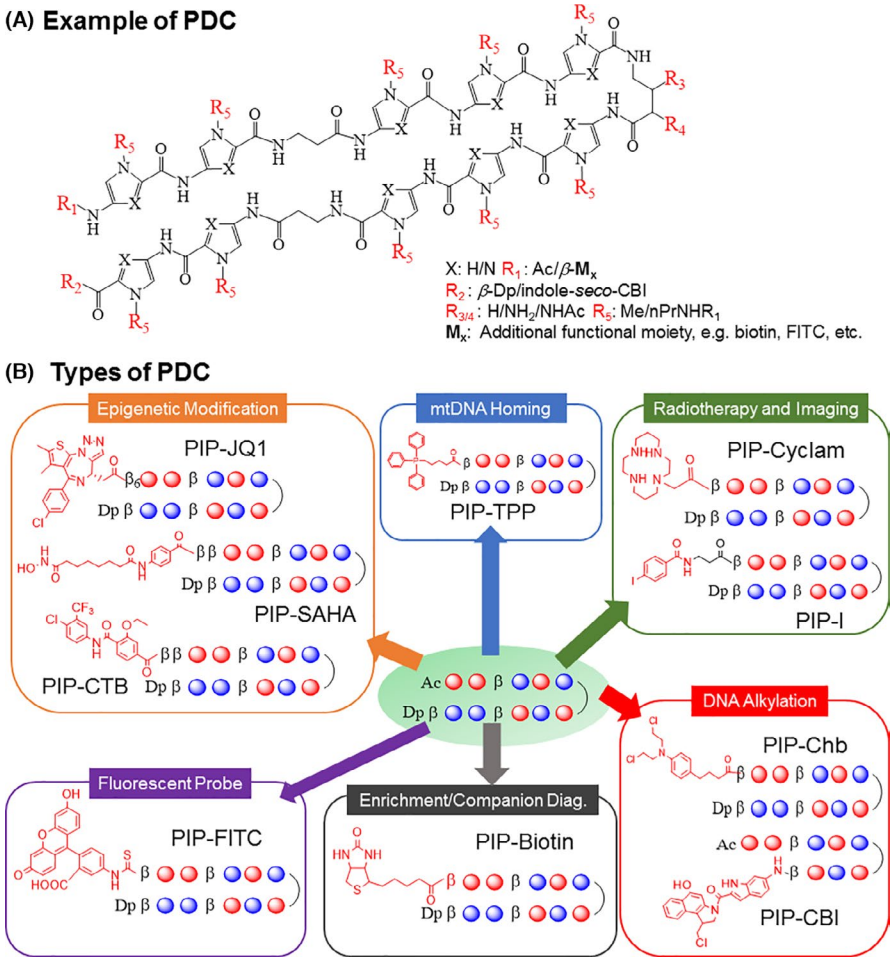

FIGURE 4 PI polyamide-drug conjugates (PDC). A, Pyrrole-imidazole (PI) polyamides can be modified by conventional synthetic techniques found in polyketide and amino acid chemistry, and the introduction of substituents into various positions (R1–R5) can improve their DNA recognition or outfit the polyamide with additional functionalities, for instance the delivery of a small molecule to target a particular genomic region in situ. B, Examples of various PDCs. PDCs incorporating various functional small molecules for epigenetic modification, fluorescent probe, DNA-pull-down assay, DNA alkylation, use of radiology, and mtDNA targeting have been reported

accumulated in mitochondria in situ, then subsequently underwent rapid elimination through the Golgi/ER pathway as previously reported,[67] or possibly through non-specific mtDNA binding. The use of lipophilic cations, such as triphenylphosphonium (TPP), had been reported to generate 100-fold improvements in preferential mitochondrial homing as a consequence of the large negative electric potential of the IMM.[36] The conjugation of lipophilic cations allowed some molecules to utilize the presence of IMM electric potential and be trafficked deep into the mitochondria.[36,68] Similarly, a PI polyamide was also reported to localize in mitochondria by binding to a lipophilic cation mitochondrial permeable protein (MPP), and the targeted ND6 gene expression was successfully inhibited by binding inhibition at the transcription factor mitochondrial transcription factor A (TFAM) binding sites for ND6 gene promotion.[22] Moreover, an alkylating MMP-PIP of 8950A-Chb, which targets a nonpathogenic mutation (m.8950G>A) in HeLa S3 cells, eliminated target mutated mtDNA, although the use of alkylating agent of chlorambucil may be limited depending on specific alkylation at the adenine sequence.[69] We also discovered that non-alkylating PI polyamide-TPP conjugates (PIP-TPPs) induced sequence-specific mitochondrial dysregulation. As TPP had a lower molecular weight than MPP peptides and was widely used for mitochondrion-targeting molecules and drug candidates,[36,68] we selected and synthesized PIP-TPPs, which were found to permeate into cells without the aid of special drug delivery systems (DDS). These PIP-TPPs accumulated and localized within mitochondria for as long as 14 days post-administration. Low dose exposure of synthesized hairpin-type PIP-TPP targeting 3243G MELAS mutation for 60 days showed a total mtDNA increase, while reduction in the proportion of mutant mtDNA was seen in heteroplasmic cells. Intriguingly, apoptosis was induced in 3243G near-homoplasmic HeLa cells after the administration of CCC-018-TPP of a PIP-TPP.[59]

Several interesting new approaches have been proposed recently for new anticancer drugs to target mitochondria. Recently, an inhibitor of mitochondrial transcription (IMTs) targeting mitochondrial RNA polymerase was well tolerated and induced a strong anti-tumor response in xenografts of human cancer cells, despite a lack of pathogenic mutant mtDNA segregation.[35] Cells lacking mtDNA, which were referred to as ρ0 cells and were generated by DNA intercalators preferentially with the mitochondrial double-stranded DNA, could proliferate in culture with glucose, uridine, and pyruvate supplementation.[70] PIP-TPPs targeting mtDNA induced mitochondrial dysfunction might also be well tolerated and increased the survival in patients with cancer. Another advantage for targeting mtDNA is that a short recognition sequence alone is sufficient to achieve specificity.[71] Similar

to antibiotics (eg, netropsin and distamycin), a smaller linear type PIP-TPPs compared with the more common hairpin configuration for the nuclear genome demonstrated similar anticancer efficacy in skin permeability and oral availability. In all, PIP-TPPs can selectively recognize their mtDNA targets in a similar motif-specific manner as their nuclear counterparts and, when delivered as the conjugate with lipophilic cation, these polyamides are able to stay longer, maintain their binding to the specific locus in mtDNA, and demonstrate drug-like characteristics as viable anticancer drug candidates.

7 | MECHANISTIC INSIGHTS OF PIP-TPP INDUCED CANCER CELL DEATH

As some pathogenic mitochondria escape MQC surveillance, a counterstrategy is critical in regaining homeostasis in therapeutic approaches involving mitochondrial care. This is especially important in developmental disorders and cancer, as a maternally inherited or newly mutated mtDNA haplotype can preferentially increase its number of copies in pro-pathogenic cells due to the bottleneck of unequal cellular division, and the subsequent clonal expansion. There are also cases in which certain mtDNA are specifically selected, leading to a homoplasmic or near-homoplasmic state to obtain survival advantages.

We created mtDNA-deficient ρ0 cells from human cervical cancer HeLa cells, and fused them with enucleated fibroblasts with the mtDNA 3243G mutation; we were able to obtain approximate 55% and 82% of 3243G cybrid cells (HeLa3243G low and HeLa3243G high cells) in this procedure. When cells were treated with CCC-018-TPP[59] or the linear smaller form of CCC-020-TPP,[71] total mtDNA copies increased in a concentration-dependent manner, and the proportion of wild-type mtDNA appeared to increase gradually in long-term cultures. Furthermore, apoptotic cell morphology and suppression of cell proliferation was observed in HeLa3243G high cells by water-soluble tetrazolium (WST) assays (IC_{50} = 7 μmol/L). In this process, ROS production was initiated as early as 6 h after CCC-018-TPP administration, and mediated an acidic condition in the mitochondria of HeLa3243G cells. JC-1 dye confirmed that the magnitude of mitochondrial membrane potential was impacted, and mitophagy was promoted in mitochondria within 24 h; the gradual increase of the relative number of apoptotic cells was also observed. Apoptosis via caspase 3 activation as death protease was also promoted, and nuclear and DNA fragmentation was observed, while cells without the mtDNA mutation were unaffected.[59,71] PIP-TPPs target not only the 3243G mutation but also acquired somatic mutations were found to promote mitophagy and the subsequent apoptosis in cells with the target mtDNA mutation.

Most anticancer drugs induce apoptosis, but the evasion of such events can contribute to treatment resistance and also to carcinogenesis/progression. In fact, selected cancer cells were resistant to PIP-TPP-induced apoptosis; rather, we saw induced cellular senescence based on SA-βGal staining, as well as the enlarged and flattened cell morphology with expression of antiapoptotic genes, such as Bcl families. Senolytics of pan-Bcl-2 (navitoclax) or Bcl-xL-specific inhibitor drugs were able to induce apoptosis of those apoptosis-resistant cells in PIP-TPP combos in vitro and in the cell-line-derived xenograft mouse model.

These changes would suggest the likelihood that escaped pathogenic mitochondria with mutant mtDNA could be recognized through MPT and/or ROS overproduction and eliminated by PIP-TPP-reactivated mitophagy, a key MQC mechanism. In homoplasmic or near-homoplasmic condition cells, the elevated rate of ROS production affected cancer cell survival, even antioxidative defense mechanisms including mitophagy are activated. In heteroplasmic conditions, moderate increases in ROS production can be overcome by antioxidative defense mechanisms and initiated reactive enhancement of mtDNA replication, therefore the total number of mtDNA copies is increased and relative proportion of the mutant is reduced. Functional mtDNA mutation may have some physiological advantage for cancer cell growth, therefore the number of the mutant haplotype genome is increased; as cancer cells confer a homoplasmic state, the surrounding TME cells can also become gradually homoplasmic, transforming into a situation in which efficacies of PIP-TPP become amplified.

Mitochondria targeting in normal cells may be tolerable as the rapid adjustment of mtDNA density is typical during cell division due to developmental and energy constraints.[70,72] Mitochondria targeting small molecules and PDCs also often showed no effect on cell survival[22,28,35] while, in certain cancers, ROS overproduction and/or functional deficiency of mitochondria did induce cytotoxicity. PIP-TPPs may also add additional functional roles in transcriptional inhibition for IMTs or MITO-PIPs.[22,35] Although the IC_{50} of PIP-TPP may be slightly higher for systemic administration, PDC should overcome this problem by representing EPR-like accumulation in tumors.[62,64] Taken together, therapy based on PIP-TPP's ability for mtDNA homing takes on a promising improved specificity for cancer and TME cells within whole tumor masses, especially for advanced cancers with homoplasmic mutations, as well as other mitochondrial and aging diseases in the form of treatment and preventative care.

8 | FUTURE PROSPECTS: mtDNA BINDERS AS A TUMOR-AGNOSTIC TREATMENT

ROS generation of mtDNA mutations contributed to tumor progression by enhancing the metastatic potential of tumor cells and susceptibility to diabetes and lymphoma development in aged mice.[12] Those pathogenic mutations were often homoplasmic and therefore appropriate therapeutic targets for PIP-TPP-induced cancer cell death.[13] However, mtDNA mutations vary and efforts required in the current paradigm of drug development is arguably costly and time consuming. Although most acquired mutations in mtDNA are passenger and heteroplasmic, hotspots and certain frequencies of homoplasmic

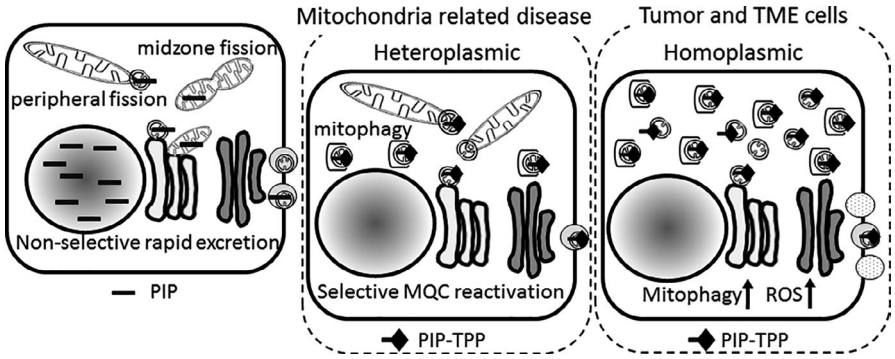

FIGURE 5 Pyrrole-imidazole polyamide-triphenylphosphonium conjugates (PIP-TPP) modalities. While PI polyamide without TPP can bind mtDNA, and the polyamide is rapidly excreted and may interact with mutated and non-mutated mtDNA without discrimination (left). PIP-TPP binds mutant mtDNA and promotes selective mitophagy of MQC within tolerable level of ROS production in heteroplasmic cells are likely to be found in mitochondria and/or age-related diseases (center). In homoplasmic cancer and TME cells, MQC survey mutated mtDNA, and promote mitophagy and associated ROS overproduction, resulting in subsequent cell death (right)

mutations have been identified, with predominant G>A and T>C substitutions across tumor types in comprehensive mtDNA analysis in human cancer.[73,74] This suggested that a common homoplastic hotspot substitution mutation in most cancers may be targeted and cleared by a PIP-TPP or a consensus target from several substitution mutation sequences. Susceptible gene polymorphisms related to cell proliferation (SGP-CP) also exist in the mitochondrial genome with a high frequency in the human population (98%-99%)[75,76]; some mtDNA SGP-CPs are homoplasmic in almost all cancer types, suggesting that mtDNA SGP-CP must contribute to tumor progression and directly affect tumor cells as well as tumor microenvironment cells, and should be an anticancer drug target for many types of cancer. We recently designed and synthesized a drug candidate of a PIP-TPP conjugate that targeted SGP-CP. Preliminary results suggested that the polyamide exhibited specific target DNA binding and could disrupt mitochondrial membrane potential after ROS production. Subsequent mitophagy and cellular apoptosis/senescence was induced in homoplasmic SGP-CP cancer cells with minimum adverse effects in mice. We believe that this novel modality of anticancer strategy using PIP-TPP targeting SGP-CP should allow us to develop a new breakthrough agnostic drugs for cancer patients for treating all homogenic cells in the heterogeneous cell population of their TME. Although substantial amounts of further research are necessary, the strategy of targeting the extremely short genomic mtDNA of 16.5 kbp by PIP-TPP without any other DDS is highly promising for homoplasmic cancer, as well as for heteroplasmic childhood mitochondrial diseases and disorders related to aging at low doses, evading the effect to normal cell (Figure 5). It is inevitable that a paradigm shift in the future can open up great possibilities such as the development of treatments with few side effects for diseases such as cancer, lifestyle-related diseases, neurodegenerative diseases, and aging.

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DISCLOSURE

The authors have no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Acquisition of data: all authors; analysis and interpretation of data: KT, TW, and HN; manuscript preparation: KT, JL, and HN.

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