OVERCOMING CHEMO-RESISTANCE USING GENETIC AND PHYTOCHEMICAL APPROACHES: BEACON OF HOPE

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Abstract:

Cancer is one of the leading causes of death worldwide, taking a toll of killing 9.6 million people annually. Chemotherapy is considered as one of the primary therapies, which is known to enhance the overall survival rates in cancer patients. The key limitation of the success of the chemotherapeutic modalities stems on acquired/*de novo* resistance in cancer, which arises through tweaking multiple alterations in genetic makeup and pro-oncogenic processes. Mounting evidences from our and other labs showed that (1) pharmacological targeting of genetic weaknesses and (2) phytochemicals-based modulations of multiple pathways hold promise for overcoming chemoresistance in cancers and therapeutic outcomes. This review highlights some of these recent findings for genetic and phytochemical based approaches to overcome chemoresistance in cancer, which may attain a special place in precision cancer therapy under clinical settings.

1. Introduction:

In addition to surgery and radiation therapy, chemotherapy represents an important therapeutic modality, which utilizes various drugs to mitigate the cancer progression. Conventionally, chemotherapy agents primarily target either the synthesis of biological macromolecules (DNA, RNA and protein) or their molecular functions, leading to demise of the cancer cells. Most commonly used chemotherapeutics include platinum, taxanes, cyclophosphamide, doxorubicin, inhibitors for Topoisomerases and PARPs etc. Mounting evidence showed that survival rates of cancer patients are dramatically increased with the advent of advanced chemotherapeutic and neoadjuvant modalities over the years. However, the benefits derived from these treatments are limited, and resistance occurs frequently[1]. Cancer associated deaths mainly mounts from the chemotherapy associated resistance, Hence, further extensive research is warranted to explore newer and effective therapeutic strategies to address the evasion of drug response through *de novo* and acquired resistance in cancer.

It is well established that genomic instability is the primary cause of cancer, where deregulated DNA repair process is associated with the sustenance of highly proliferative cancer cells[2]. Genomic instability arises due to frequent rearrangements, gene copy number changes, and mutations in cancers. Interestingly, it is also known that these genetic defects contribute resistance to chemotherapeutics, leading to differential response and therapeutic failure in different cancer patients. Incidentally, these vulnerabilities that are specific to cancer cells, making them potential targets for cancer therapy for overcoming resistance[3]. Hence, there is burgeoning research interests in investigating therapeutic strategies to target cancers for personalized therapy. It is also interesting to note that resistance in cancers is associated with the rewiring of multiple compensatory signalling pathways and molecular processes. Since, phytochemicals possess excellent anti-cancer properties with pleiotropic effects on multiple targets, these molecules are considered as key therapeutic modalities to circumvent chemoresistance in cancers[4]. This chapter highlights a comprehensive summary of some of our and others research findings for addressing chemotherapy and chemoresistance employing genetic and phytochemical approaches. The potential of these approaches targeting specific vulnerabilities in cancers for their sensitivity/resistance hold promise and could lead to significant improvements in the management of cancer progression and chemoresistance.

2. Genetic approaches to overcome resistance in cancer:

Several major genetic mechanisms have been implicated in *de novo* and acquired resistance to chemotherapeutics. Especially, activation of critical signalling circuits, genetic and epigenetic alterations to support cell survival and oncogenic growth. With the advancements in genome sequencing techniques, tumor specific genetic alternation and their critical association with tumor progression are increasingly evident. Understanding such specific weaknesses is essential (1) for selective targeting tumor, leading to synthetic lethality and (2) to reduce *de novo* and acquired resistance with concomitant enhancement of tumor sensitivity to chemo/radio-therapy[2].

2.1 Synthetic lethality approach for targeting chemoresistance:

Classically, the concept of synthetic lethality involves indispensable role of a pair of genes or proteins, where functional inactivation of one gene along with the simultaneous pharmacological targeting of the other triggers hypersensitivity in cancer cells with minimal effects on normal cells (with functional genes)[5]. Classical instance for synthetic lethality by targeting BRCA1/2 negative breast cancer cells with PARP inhibitor is successfully used in clinical setting[6]. Working in similar line, in 2019 three independent reports revealed synthetic lethality in cancers with higher microsatellite instability (MSI), when WRN RECQL helicase is silenced or knocked out[7-9]. MSI arises due to compromised DNA mismatch repair. Moreover, extensive expansion of (TA)n-repeats, triggers genome wide accumulation of non-B form DNA in MSI cells. Mechanistically, stalled forks at these structures elicits ATR dependent WRN functions, which indispensably resolves these structures through its helicase activity. Besides, it is known that expression of WRN is downregulated across different types of cancers, due to changes at epigenetic levels. Thus, targeting WRN in cancer cells with defective WRN expression/function can be more effective personalised therapy. Recently, we showed compromised homologous recombination (HR) repair in WRN defective cancers, which can be effectively targeted in ionizing radiation (IR) therapy[10-11]. For the first time, our work revealed a crucial role of WRN in regulating CHK1 and p38-MAPK for RAD51 assembly during HR repair, which otherwise is significantly compromised in cancer cells with defective WRN expressions[11]. Interestingly, pharmacological inhibition of CHK1 or p38-MAPK led to the hypersensitization of cancer cells to IR treatment. In corroboration with *in vitro* data, our investigation showed a remarkable sensitization of WRN-deficient melanoma tumors in syngenic preclinical mouse models, when treated with IR and CHK1 inhibitor [11]. Together these findings suggest that the WRN deficient cancer patients may respond better to combination treatment of IR with either CHK1 or p38-MAPK under clinical settings.

Another important RECQL helicase *i.e.,* RECQL5 is known to regulate transcription and DNA repair. Expression of RECQL5 is severely down-regulated in multiple cancers, including breast and gastric cancers[12]. We discovered synthetic lethal interaction of RECQL5 deficiency with checkpoint kinase 1 (CHK1) inhibitor in breast cancer (unpublished data). Our extensive investigation revealed that RECQL5 is involved in the regulating activation of a SMARCAL1 (DNA remodelling enzyme) which is required for further processing of defective replication forks, leading to sustainable repair by both Break Induced Replication (slow) and 53BP1 mediated (fast) repair pathways. Intriguingly, over-activation of SMARCAL1 was observed in RECQL5 deficient cells, leading to synthetic lethality in RECQL5-deficient breast cancers in response to pharmacological inhibition of CHK1. As unregulated fork reversal induced by CHK1 inhibition leads to generation of copious amounts of single-ended double strand breaks that are bound by 53BP1 making them difficult to be repaired. This leads to the generation of toxic DNA intermediates, which is responsible for synthetic lethality in RECQL5-deficient cancer cells under CHK1 inhibition (Figure 1). Thus, a therapeutic response to CHK1 inhibitor (under various clinical trials) may be enhanced in breast cancer patients with RECQL5-deficiency. Further, to expand the efficacy of RECQL5 and CHK1 synthetic lethality, we have also designed, synthesized and developed a RECQL5 inhibitor, named as 4a, which targets specifically RECQL5 and not any other RECQ

helicases[13]. The functional activity of RECQL5 was potently inhibited by 4a by stabilizing the complex of RECQL5-RAD51, which in turn leads to defective HR repair, accumulation of DSBs and enhanced killing of breast cancer cells with RECQL5 overexpression. Interestingly, the effects of 4a is a severely minimized in the normal mammary epithelial cells due to overall low RECQL5 expression in these cells[13]. Collectively, our extensive investigation, based on integration of multiple approaches (design, synthesis, in silico, CRISPR, siRNA silencing, ectopic expression, mutagenesis, biochemistry and *in vivo* tumor models *etc*) revealed that compound 4a is selective inhibitor of RECQL5 and may be used as a single agent to kill RECQL5-expressing breast cancers[13]. Moreover, 4a can also be used as an adjuvant with cisplatin to impair RECQL5-mediated HRR, hence it may reduce cisplatin resistance in breast cancers (Figure 1)[13]. Based on our investigation[13], several companies have commercialized 4a, as a specific RECQL5 inhibitor, for the R&D purposes (https://www.medchemexpress.com/recql5-in-1.html; https://www.glpbio.com/recql5-in-1.html).

PARP inhibition leads to synthetic lethality or hypersensitivity in cancer cells with defective homologous recombination repair (HRR)[14]. For the first time, we have shown that the combination of PARP1 inhibitor (PARPi) with RECQL5 inhibitor (4a) leads to increase sensitivity of HR-proficient breast cancers by significantly reducing *de novo* resistance[15]. 4a impairs homologous recombination by stabilizing the physical interactions of RECQL5 and RAD51 complex and hence enhances sensitivity of multiple breast cancer cell lines to PARP inhibitor treatments. Mechanistically, PARP inhibition led to replication stress which essentially requires HR for the repair. We showed that inhibition of RECQL5 by 4a led to robust replication stress and accumulation of extensive DSBs in response to PARPi. Imperatively, impairment of HRR through inhibition of RECQL5 provoked NHEJ mediated repair, mitotic catastrophe and sensitivity of breast cancer cells to PARPi (Figure 1) [15]. One of the concerns of PARPi based therapy is the occurrence of metastasis. Inhibition of RECQL5 functions by 4a significantly supresses metastatic potential associated with PARPi. Together, we identified RECQL5 as a novel pharmacological target for expanding PARPi based treatment horizon for HR-proficient cancers (Figure 1)[15]. Of note, the combination treatment of PARPi and 4a has lesser effects on normal mammary epithelial cells, which expresses low RECQL5 *vis-à-vis* breast cancer cells[15].

2.2 Targeting DNA repair deficiency to counter chemoresistance to Topoisomerase 1 inhibitors:

Topoisomerases are essential enzymes which deal with topological problems on the DNA, stem from fundamental molecular events such as replication, transcription and DNA repair which are absolutely necessary for survival[16]. The basic function of topoisomerases essentially entails relaxation of DNA supercoils through a strand-cleavage-passage-religation cycle. The strand cleavage reaction is a transesterfication reaction that involves formation of TOP1-DNA covalent complexes (TOP1ccs). The covalent cleavage intermediate, whereby the 3'-phosphate group of TOP1 protein is linked to the substrate DNA, of TOP1 requires proper alignment with the 5' OH group in order for efficient religation[17]. TOP1ccs, if stabilized, can rapidly become sources of potentially lethal double strand breaks, arising out of collision with oncoming replication forks, or endonucleolytic intervention at sites of R-

loops which accumulate in the vicinity of TOP1ccs[18]. The intrinsic vulnerability of TOP1ccs makes them an attractive therapeutic target.

Consistent with its indispensable role in many molecular pathways, TOP1 has gaianed intense research interests for the possibility of its pharmacological targeting in cancer therapy. Since the discovery of camptothecin in the 1950s[19], there have been continuous efforts aimed at the development of clinically relevant TOP1 inhibitors (resulting in FDA approved drugs *e.g.,* Topotecan and Irinotecan, and understanding their role in stabilizing TOP1ccs at cellular levels. However, cancer cells develops multiple resistance processes to TOP1 inhibitors. These include degradation of TOP1ccs through protesome dependent and independent pathways. Tyrosyl-DNA phosphodiesterase 1 (TDP1) removes the covalently linked degraded TOP1cc[20-21]. Alternatively, TOP1ccs may attract multiple endonucleases (*e.g.,* XPF-ERCC1, Mus81-Eme1, Mre11 or CtIP, with follow up nucleotide excision repair. Recently, we showed that WRN RECQL helicase plays a key role in recruiting endonucleases to remove TOP1cc[25] At the cellular and physiological levels, classical TOP1 poisons like camptothecin are inactivated through interaction with serum albumin. Inactivation of irinotecan by UGT1A, CYP3A4 and CYP3A5[22], reduced cellular accumulation due to dysregulation of one or more organic cation transporter, or ABC transporter mediated efflux of TOP1 poisons[23]. Further, a large array of TOP1 mutations and post-translational modifications have been reported which affect both interaction of TOP1 with drugs as well as cellular processing of TOP1ccs[24].

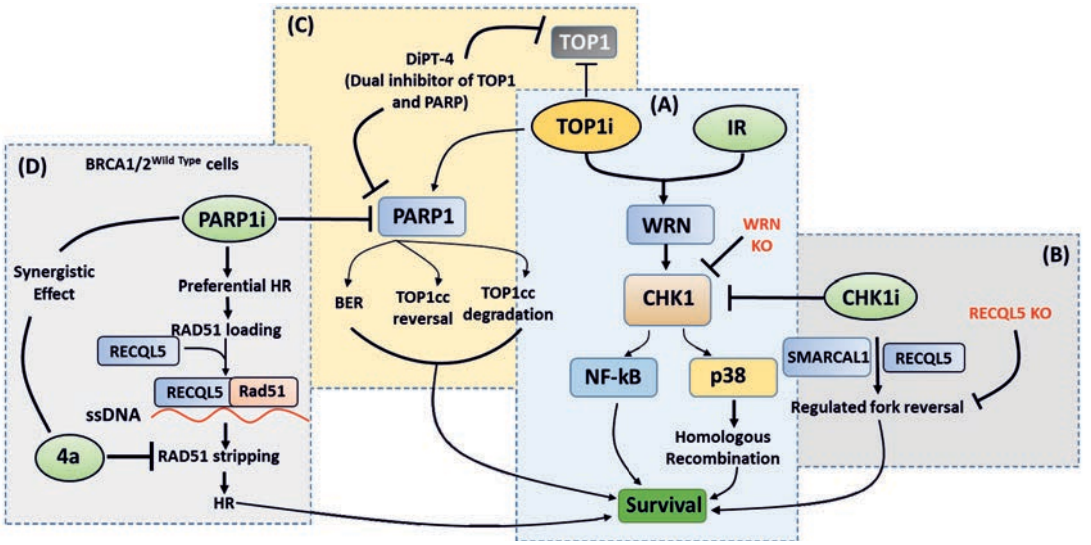

Figure 1. Role of WRN, RECQL5, TOP1 and PARPs in chemo-resistance, and their targeting by pharmacological inhibitors for enhancing chemo-sensitivity in cancers.

Besides, NF-κB is a key prosurvival transcription factor, which plays a crucial role in resistance to various chemotherapeutics and radiation therapy. These chemotherapeutic and radiation treatments generate the double strand break (DSBs), which activate the canonical NF-κB pathway[25]. However, drugs like TOP1 inhibitor (camptothecin, CPT) at physiological

relevant dose (nanomolar concentration), does not generate DSBs but causes genotoxic stress[26]. Recently, we have shown a causal link of TOP1cc processing by WRN and its association with NF-κB associated chemoresistance to TOP1 poisoning. In this investigation, our whole genome analysis with ~25000 genes showed an hitherto unknown link of TOP1 inhibitor mediated activation of pro-survival pathways, especially NF-κB, with therapeutic resistance to TOP1 inhibitors[27]. Mechanistically, we found WRN (RECQ helicase) orchestrates TOP1cc complex removal through proteosome dependent and independent process, leading to robust enhancement of single strand DNA (ssDNA) generation. Further, ssDNA activates ATR-CHK1 pathway which involves WRN, leading to activation of NF-κB. We have also shown that WRN deficient cells are defective in activation of ATR-CHK1 pathway and NF-kB process, leading to their hypersensitivity to CPT (Figure 1) [27]. Since in different types of cancers (colorectal cancer, breast cancer etc), WRN expression is severely compromised because of epigenetic silencing[28], targeting these tumors with TOP1 inhibitors may help in achieving better outcomes for personalized/targeted therapy. Intriguingly, helicase dead and/or exonuclease dead mutant of WRN (WRN^{E84A}, $WRNK^{577M}$, and $WRN^{E84A-K577M}$) were equally effective in the TOP1cc removal, ssDNA generation and signaling for NF-κB activation, suggested an important non-enzymatic function of WRN in TOP1cc processing and therapeutic resistance to CPT[27]. In corroboration with patient data and above results, our preclinical evaluations showed that melanoma tumors with depleted WRN were highly sensitive to TOP1 inhibition *in vivo* mouse model. Together, our investigations with multiple approaches identify the hitherto unknown association of cancer resistance to TOP1 inhibitors with non-enzymatic WRN RECQL helicase, while offering avenue for potential targeted therapy for WRN-deficient cancer patients (Figure 1)[27].

Another recent investigation of our laboratory has demonstrated an important development of dual PARP-TOP1 inhibitor, which showed attractive therapeutic strategy to target cellular resistance to TOP1 inhibitors[29]. PARP1 is known to have an instrumental role in molecular processes in response to TOP1ccs. Drug-induced stabilization of TOP1ccs has been shown to induce rapid PARylation of TOP1 and TOP1ccs. In addition, PARylation has been shown to facilitate TOP1cc resolution through a multifold mechanisms, including direct PARylation mediated facilitation of TOP1cc religation (unless TOP1 is poisoned), and recruitment a plethora of repair proteins at the site of TOP1ccs (*e.g.,* TDP1, XRCC1 and Ligase III etc.). Hence, PARP mediated recruitment of repair proteins plays a pivotal role in resolution of drug-stabilized TOP1ccs[30-31]. Consequently, a large of body of information exists in the favour of developing combinatorial therapeutic regimens involving PARP inhibitors and TOP1 poisons like irinotecan or topotecan. Although promising results have been obtained in *in vitro* and preclinical scenarios[32], clinical trials have yielded modest results, owing to severe dose limiting toxicities[33-34]. Dual inhibitors often offer significant advantages over combinatorial regimens, especially with respect to pharmacokinetic incompatibility between individual agents.

Drawing from our in-house expertise in organic synthesis, we attempted to develop a single agent based dual inhibitor of PARP and TOP1, utilizing pharmacologically relevant pharmacophores of Olaparib (and FDA approved PARP inhibitor) and 1,8-Napthalamide (an investigational TOP1 inhibitor). We synthesized a library of 11 compounds (named as dual

inhibitor of PARP and TOP1, DiPT-1 to -11) with minor variations in side chains and the linker between the two pharmacophores[29]. Large scale screening against multiple cell lines revealed one compound (DiPT-4) displayed significantly improved cytotoxic potential compared to Olaparib as well as 1,8-Naphthalimide. Employing MCF-10A cells, we further demonstrated that DiPT-4 is selectively cytotoxic to cancer cells, while sparing cells of non-cancer origin. *In vitro* assays revealed promising inhibitory activity of DiPT-4 towards both TOP1 and PARP1; in addition, *in silico* analysis revealed the significant binding activity of DiPT-4 to the active sites of PARP1 and TOP1. DiPT-4 induced DNA damage and TOP1cc stabilization inside cells[29]. Interestingly, although DiPT-4 induces TOP1cc formation, cellular PARylation was significantly lowered as compared to classical camptothecin induced TOP1 poisoning. Furthermore, DiPT-4-mediated TOP1cc stabilization did not trigger TOP1cc degradation, which was attributable to inefficient PARylation of TOP1ccs[29]. In summary, our results demonstrate a proof of concept for further investigations into more promising single molecule simultaneous targeting of TOP1 and PARP1; this in turn may have significant potential for overcoming therapeutic outcomes of TOP1 mediated resistance and improving targeted therapies (Figure 1) [29].

2.3 Overcoming resistance to PARP inhibitors using phytochemicals:

Poly (ADP-ribose) polymerases inhibitors (PARPi) are an important class of drugs that are currently in usage as targeted chemotherapeutic agents. These are currently approved for certain groups of patients bearing hereditary breast and ovarian cancers which are homologous recombination (HR) repair deficient[35]. PARPi inhibit PARylation, a post translational modification carried out by a group of proteins referred to Poly (ADP-ribose) polymerases, majorly PARP1 and PARP2. These PARPs play an important role in replication, DNA damage repair, maintenance of genomic stability and chromatin remodelling among other vital functions in the cells[36].

Sensitivity of BRCA1/2 deficient cancer cells to PARPi were initially proposed on the basis of a concept referred to as synthetic lethality[35,37]. Later, the concept was extended to include HR deficient tumours as it was observed that these were also highly sensitive to PARPi. Intriguingly, certain cancer cell lines which are HR proficient respond well to PARP inhibitors while HR deficient cancers are resistant to PARPi treatment [38]. These findings lead to a complicated view of the mode of action of PARP inhibitors and hence are active topics of research. Further understanding of cancer sensitivity and resistance to PARPi led to the elucidation of multiple mechanisms of resistance to PARP inhibitors. Reversion of homologous recombination was a common theme which led to the resistance to PARPi[39]. Additionally, stabilization of replication fork[40], loss of 53BP1[41], activation of residual HR pathways, shieldin protein complex[42] *etc* were found to confer resistance to cancer cells against PARPi mediated insults.

Research from our lab[43] and that of others[44-45] has shown that autophagy, majorly a cytoplasmic process, plays a very important role in determining sensitivity of BRCA-WT breast cancers to PARPis. Cells deficient in BRCA1 inherently had an upregulation of autophagy, indicating that autophagy may be a compensatory mechanism activated in the absence of functional HR pathway and probably aiding in mitigating the PARPi mediated damage in the cells[44]. Our systematic analysis unravelled that autophagy indeed conferred

PARP inhibitors mediated *de novo* resistance in breast cancers cells[43]. Talazoparib, a third generation PARPi, was found to extensively induce autophagy in breast cancer cells. Multiple cell-based assays like p62 and LC3 colocalization analysis, GFP-LC3 foci formation, tf-LC3 based traffic light assays among several others indicated that talazoparib robustly activated autophagy and pointed at elicitation autophagic flux in the breast cancer (Figure 2) [43]. Enhanced induction of cell death was observed in autophagy deficient breast cancer cells treated with talazoparib[43]. These results indicated that PARPi activated autophagy was leading to *de novo* resistance to PARPis. Our data showed that HR proficient *de novo* resistant breast cancers were successfully targeted with talazoparib (PARPi) using the autophagy inhibitor chloroquine as an adjuvant (Figure 2) [43]. Results indicated that the concomitant treatment of talazoparib and chloroquine abrogated the cytoprotective autophagy, and enhanced DNA damage and activated deleterious non-homologous end joining (NHEJ) pathway eventually inducing apoptosis and mitotic catastrophe (Figure 2) [43]. Our results also hinted at an excessive genomic instability induction in the simultaneous targeting of PARPi and autophagy in breast cancers.

In another related work, we reported the targeting of cytoprotective autophagy induced by talazoparib by using another phytochemical, resveratrol (Figure 2) [46]. Resveratrol is a natural polyphenol found in many foods like peanuts, grape-wine and others. Multiple reports have previously shown resveratrol to induce autophagy with different molecular mechanisms like inhibition of SIRT1, inhibition of mTOR kinase etc. There are several other conflicting evidences suggesting resveratrol mediated inhibition of autophagic pathway in cells[46]. Since resveratrol is a natural molecule with multiple molecular targets, it is likely that the observation of induction or abrogation of autophagy thereof might be highly context dependent. In our work, we found that talazoparib with resveratrol co-treatment induced synergistic cell death in breast cancers[46]. While resveratrol alone significantly induced upregulation of autophagic markers in the cell, the completion of autophagy *i.e.,* autophagosomes-lysosome fusion, was inhibited in the presence of resveratrol (Figure 2). Moreover, in the presence of talazoparib induced high autophagic flux, resveratrol was able to prevent the autophagosome-lysosome fusion conferring sensitivity to PARPi treatment and inducing cell death[46]. Resveratrol was able to induce extensive lysosomal membrane permeabilization (LMP) which acted to prevent autophagic flux in breast cancer cells [46]. In both the studies discussed above[43,46], we found that the combination of talazoparib with autophagy inhibitor could effectively reduce the tumour volume while causing minimal toxicity in the SCID mice tumour xenograft models.

Replication stress has long been considered as an *Achilles heel*, an inherent vulnerability in multiple cancers due to the excessive growth rate of the tumour cells. Replication stress induction has been considered one of the major targets that could lead to better therapeutic outcomes in cancers. While PARP inhibitors invoke significant replication stress in cancers[48], *de novo* resistance with several DNA repair and replication stress response related mechanisms tip the balance in favour of cancer survival. By enhancing the replication stress in cells, the inherent threshold could be breached and cancer cells could be targeted to induce cell death. A resveratrol analogue 4,4'-dihydroxystilbene (DHS) has been found to induce extensive replication stress due to inhibition of M2 subunit of ribonucleotide reductase, an important enzyme in DNA replication[49]. Currently, our work focuses on the synergistic

activity of the phytochemical DHS with PARPi, talazoparib in ovarian cancers. We have found that DHS combined with talazoparib induces extensive DNA damage and thereby replication stress (Unpublished work). It also leads to the formation of single stranded DNA and protracted residence and accumulation of cancer cells in the S-phase of the cell cycle, eventually leading to replication catastrophe in the SK-OV-3 cell line model. The combination effects were also successfully replicated in SCID mice xenograft models of ovarian cancer with minimal toxicity in pre-clinical models (Unpublished work).

While efficacy has been up to the mark, therapy-associated adverse effects is the major challenge linked with the usage of autophagy inhibitors and replication inhibitors in the clinical scenario. Natural molecules and/or phytochemicals are therefore the way ahead as they offer the necessary efficacy with the least side effects. In this regard, our data indicate that phytochemicals- resveratrol, chloroquine and DHS may be considered for usage as adjuvants to ameliorate the resistance associated with PARP inhibitor therapy.

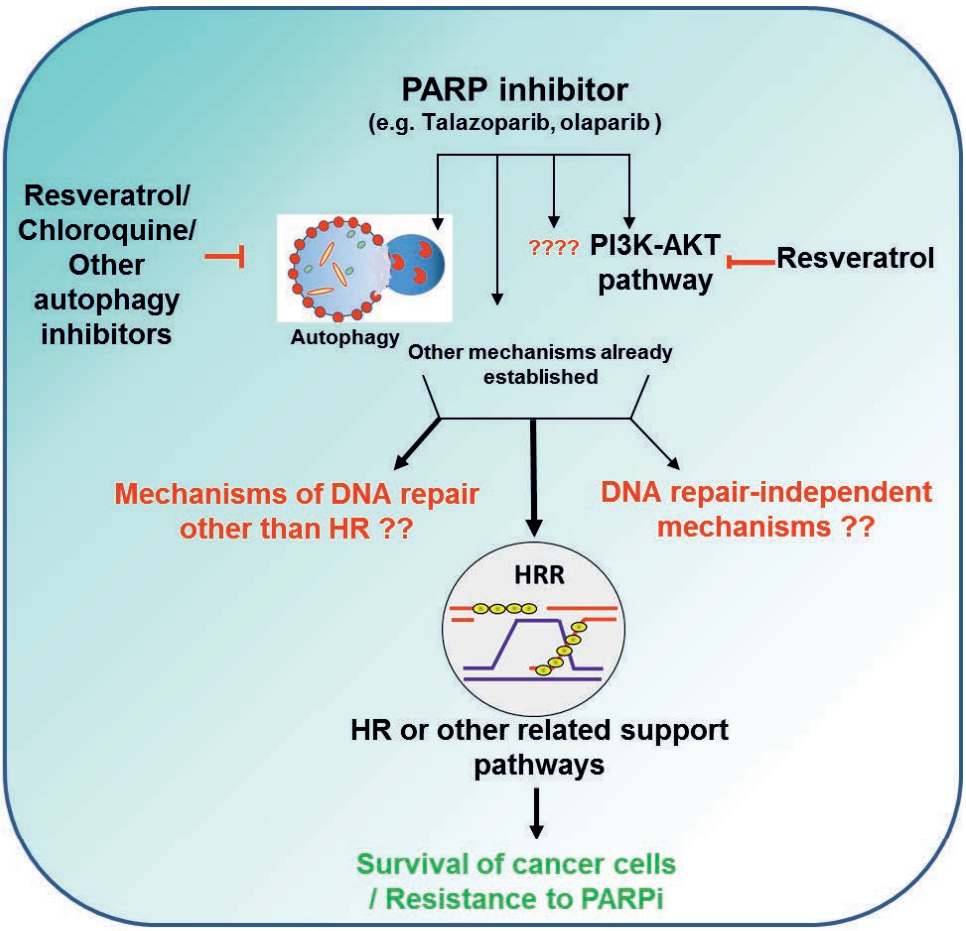

Figure 2. Chemoresistance processes to PARPi and its mitigation by resveratrol and other autophagy modulators.

3. Phytochemicals for targeting chemoresistance:

Pro-onocogenic process mediated chemoresistance is strongly associated with many key proteins like STAT3, p53, AP-1 (c-JUN/c-FOS), NF-κB, c-MYC [50]. Targeting these prooncogenic proteins or activation of proteins involved in cell death process are believed to improve the chemotherapy outcome[51]. Of note, many pharmacological inhibitors of these survival proteins have limited clinical acceptability due to their inherent systemic toxicity. In this regard, extensive research on dietary phytochemicals and epidemiological evidence showed the dietary influences on cancer prevention, and effects of phytochemicals on multiple molecular targets in cancer with minimal side effects[52]. Importantly, half of the FDA approved chemotherapeutic drugs are derived from natural products[53]. Among dietary phytochemicals, spice derived agents have a special place in suppressing the cancer associated transformation, hyper-proliferation and inflammatory processes[54]. Here, we outline our research findings, showing potentials of some of the phytochemicals and their derivatives in reducing progression and metastatic potentials of tumors.

3.1 Malabaricones deregulate redox homeostasis for inhibition of cancer growth:

Earlier, we demonstrated the isolation of malabaricone C (a phenolic compound found in *Myristica malabarica*, an Indian spice). and explored its superior radical scavenging and antioxidant activity in comparison to curcumin[55].In the presence of Cu(II), Mal C was found to induce ROS-mediated DNA damage[56] and lysosomal membrane permeabilization in cancer cells[57]. We also showed that mal C has the ability to kill human lung cancer cells through induction of DNA damage and ATM/CHK1-p38-mediated mitochondrial death[8]. It is well known that reactive oxygen species (ROS) levels is constitutively up-regulated and hence provides an opportunity to target redox homeostasis in malignant cells. To this end, we have shown that mal C generates copious amount of ROS while reducing intracellular GSH. Intriguingly, only thiol-antioxidants (NAC/GSH) restituted intracellular GSH level but paradoxically enhanced DNA DSBs and apoptotic cell death induced by mal C[59]. Our results revealed this thiol-antioxidant based sensitization is based on two tightly coupled biochemical processes. Firstly, mal C undergoes “catechol-quinone redox cycle” of mal C, in the presence of thiol antioxidants, and induces enhanced amounts of ROS and DNA damage. Secondly, mal C causes oxidation of many transcription factors like [p53, p65 (NF-κB) etc.], which in turn are glutathionylated in the presence of NAC. Gulatathionylated transcription factors are retained in the cytoplasm and hence their protranscription functions are abrogated[59]. Furthermore, cytoplasmic accumulation of glutathionylated p53 led to its accumulation in mitochondria and enhancement of mitochondrial death process in cancer cells (Figure 3). The preclinical results, obtained from two independent tumor models in mice support our conclusion that NAC can further sensitize and enhance the anti-tumor effects of mal C *in vivo*[59].

3.2 Targeting cisplatin resistance in cancer cells by salinomycin:

Platinum-based chemotherapy regimens (cisplatin/cis-diamminedichloro-platinum (CDDP), carboplatin plus other cytotoxic agents) are considered the standard treatment for patients diagnosed with bladder, lung, head and neck, testicular, ovarian, and advanced breast cancer

with metastasis. However, due to the emergence of cisplatin resistance and severe side effects in non-malignant tissue, there is a need to understand the molecular mechanisms of CDDP resistance and its mitigation by combination therapy[60]. Several molecular targets like BCL2, cyclin D1, and NF-κB are reported to play pivotal roles in the CDDP resistance[61]. Among them, NF-κB (a transcription factor) is considered one of the major players. It promotes drug resistance by offering an alternative survival mechanism, achieved through the upregulation of anti-apoptotic proteins[62]. Thus targeting/blocking NF-κB might increase the efficacy of cancer drugs not just in CDDP-resistant cancer cells but also in cancer cells showing resistance against radiation and other commonly used chemotherapeutic-drugs, including paclitaxel, gemcitabline, doxorubicin (adriamycin) and vinblastine[63-64].

Salinomycin is an antibiotic from *Streptomyces albus*, and known to significantly reduce the breast cancer stem cells fractions 100 times more efficiently than paclitaxel[65]. We explored its ability to combat chemo-resistance to cisplatin[66]. Initially, we generated CDDP-resistant breast cancer cells (i.e.MCF7DDP) by treating MCF7 cells with a repeated dose of cisplatin for 4-6 weeks. This was done to a ensure similar treatment conditions as given to patients during cisplatin therapy. In our assays, salinomycin inhibited the cell viability of these CDDP-resistant breast cancer ($MCF7^{DDP}$) cells in concentration-dependent manner[66]. Using phase contrast microscopy, we showed that salinomycin induced significant morphological changes like cell shrinkage and membrane blebbing (a marker for apoptosis). Using clonogenic assay, salinomycin was found to reduce the reproductive ability (cell division) of $MCF\text{-}7^{DDP}$ cells. Our results also demonstrated that after salinomycin treatment, CDDP-resistant breast cancer cells underwent significant cell cycle arrest, DNA damage, and diminished metastatic migration[66]. Mechanistically, approx. 2-fold higher concentration of p65 (an important subunit of NF-κB) and its downstream proteins like survivin, XIAP, and BCL2 were observed in CDDP-resistant breast cancer (MCF7DDP). Interestingly, salinomycin abrogated the expression of all these effectors. Together, our results indicated potential use of salinomycin in overcoming *de novo* and/or acquired chemo-resistance in cancer cells to cisplatin therapy (Figure 3) [66].

3.3 Overcoming chemo-resistance by autophagy modulators:

Autophagy is a fundamental process, preserved throughout the evolution, in which the cell sequesters cellular components in autophagosomes and degrades them *via* fusion with lysosomes in a regulated manner. Intriguingly, cancer cells can alter autophagy to provide energy, macromolecules, to sustain the stemness of cancer stem cells, promote epithelial-mesenchymal transition, oxidative stress, *etc.*[67]. In certain cancers, *i.e.,* pancreatic ductal adenocarcinoma, autophagy and associated lysosomal processes are crucial drivers of aggressive cancer progression which aid in sustenance and meeting the growth requirements in the hostile tumour micro-environment[68-69]. It is interesting to note that autophagy is one of the primary forces behind cancer cells quick adaptation and a major contributor to their resistance to various therapeutic agents[70]. Additionally, it has been discovered that autophagy is crucial for the reactivation of dormant cancer cells responsible for the recurrence of cancer after therapy. A myriad of evidence shows that targeting autophagy (with chloroquine, resveratrol *etc*) along with other chemotherapeutics like doxorubicin[71], imatinib[72],

gemcitabine, PARPi[43,46], docetaxel[73], panobinostat[74], MEK1/2i[75], provides better therapeutic outcomes.

Multiple efforts and strategies are being made to therapeutically target autophagy in cancers at various stages of the autophagic process or lysosomal function in cancers[76]. Although one of the most promising drugs, chloroquine (CQ), damages lysosomes, its therapeutic applications are severely constrained because of its higher physiological concentration and greater systemic toxicity. These issues warrant investigations into and development of novel autophagy inhibitors and/or modulators with fewer side effects. In this regard, phytochemical-based autophagy modulators can be ideal choice considering their probable lower side effects. Recently, a number of phytochemical-based compounds such as periplocin, leelamine[77], jolkinolide B[78], PC3-15[79], pristimerin[80], Phloretin[81], Formononetin[82], etc. have been tested for targeting autophagy and lysosomes in cancer cells.

3.3.1 Malabaricone derivatives as autophagy modulators:

Since mal C has the potential to induce lysosome membrane permeabilization in cancer cells, we attempted to synthesize a series of mal C analogues in the search of potent autophagy deregulating molecules. Amongst these analogues, one of the mal C derivatives, named as ML-9, found to supresses cancer growth efficiently through abrogation of autophagic flux (unpublished data). Our results revealed that ML-9 also radiosensitizes cancer cells through enhanced DNA damage. Mechanistically, ML-9 treatment led to the loss of lysosomal membrane permeability (LMP), leading to accumulation of autophagosomes. Decreased p62 is an indicator of successful autophagy while p62 aggregates are identified as indicator of unfolded/inactive proteins. We observed p62 accumulation in the cytoplasm of ML-9 treated-cancer cells, indicating stress induced unfolded/inactive proteins unable to be cleared by autophagy. Collectively, our investigation demonstrated ML-9 as a potent inhibitor of tumor growth progression and autophagy flux, which may enable reduction chemo/radioresistance in cancers (Figure 3).

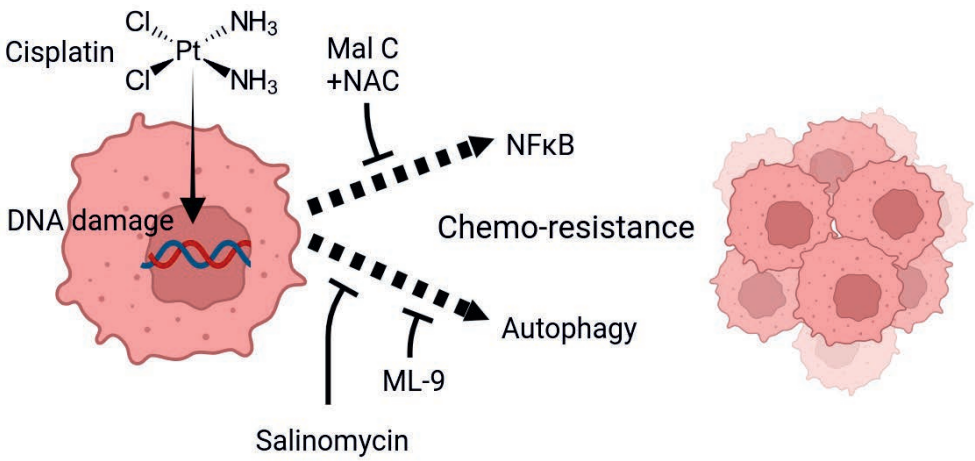

Figure 3. Major chemoresistance processes *e.g.*, Nf-kB and autophagy and their modulations by phytochemicals.

3.3.2 Natural stilbene molecules are excellent autophagy modulators:

We have shown that resveratrol and *trans*-4,4'-dihydroxy stilbene (DHS), anti-cancerous agents from the stilbene family, target and disrupt lysosomes[46,83] and also cause inhibition of autophagosome fusion with lysosomes[46]. However, due to pleiotropic effects of stilbene molecules, its efficacy as lysosome specific targeting is limited. In order to target stilbene to lysosomes in cancer cells, we have carried out in-house synthesis of a series of novel rationally designed conjugates of pharmacophores of CQ and DHS. These lysosome targeting stilbenes are named as lysostilbenes. Our investigation showed that one of the lysostilbenes, LS-3, inhibits growth of pancreatic ductal carcinoma (PDAC) at nanomolar concentration while it has minimal cytotoxicity toward normal cells. LS-3 specifically targets lysosomes and causes lysosome membrane permeabilization (LMP)-mediated apoptotic cell death in PDAC. Interestingly, LS-3 was found to inhibit autophagy-mediated clearance. Furthermore, taking into account the role that transcription factor EB (TFEB), a master regulator of the lysosome and autophagy genes the LS-3 effect was explored on TFEB dynamics and transcriptional activity. LS-3 was found to cause increased nuclear accumulation and transcriptional activity of TFEB. Furthermore, we demonstrated that CRISPR-mediated knockout of TFEB leads to hypersensitization to LS-3 treatment.

Taken together, our extensive investigations along with that of others highlight the efficacy of phytochemical modulators of autophagy for targeting chemoresistant tumours. Lysostilbenes (a phytochemical-based conjugate) and other novel autophagy inhibitors like chloroquine derivatives may be used for targeting autophagy/lysosome, a key mechanism responsible for chemoresistance in PDACs and other cancers. These advancements provide a strong rationale for further investigation and evaluation of phytochemical based autophagy inhibitors for anticancer therapy.

3.4 Targeting pancreatic cancer through Hydroxychavicol:

Pancreatic cancer is one of the most lethal cancers, with poor survival and prognostic outcomes. Problems associated with pancreatic cancer therapy range from recurrent chemotherapeutic resistance to early metastasis. Work from our laboratory has demonstrated that Hydroxychavicol (HC), a natural molecule isolated from *Piper betel* leaves shows promising cytotoxic activity towards pancreatic cancer cells. This, coupled with its high therapeutic index prompted us to further investigate its molecular mechanism of action. We demonstrated that HC induces DNA damage and JNK-pathway-dependent apoptosis in pancreatic cancer cells. We also showed that HC inhibits Epithelial-Mesenchymal Transition (EMT) and represses the expression of genetic drivers of EMT. These data, taken together, makes HC an interesting candidate for future studies, as there are no clinically available anti-metastatic agents for pancreatic cancer[84].

4. Conclusions:

Current investigations sheds light on multiple mechanisms involved in the cancer chemoresistance processes in clinical settings. Recent advancements in our and other labs have identified several genetic alterations and cellular pathways, which may be targeted by modulators to achieve sensitization of tumor. Besides, combination therapy employing

chemotherapeutic agents and some of the phytochemicals described above have the potential to further improve the clinical outcomes. Current research should be focused with an ultimate goal of developing advanced chemotherapeutics, which can differentially enhance the tumor response while minimizing the adverse effects on the normal tissues within the framework of personalized cancer therapy.

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