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Review

Signaling alterations caused by drugs and autophagy

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ABSTRACT

Autophagy is an evolutionary conserved process that recycles cellular materials in times of nutrient restriction to maintain viability. In cancer therapeutics, the role of autophagy in response to multi-kinase inhibitors, alone or when combined with histone deacetylase (HDAC) inhibitors acts, generally, to facilitate the killing of tumor cells. Furthermore, the formation of autophagosomes and subsequent degradation of their contents can reduce the expression of HDAC proteins themselves as well as of other signaling regulatory molecules such as protein chaperones and mutated RAS proteins. Reduced levels of HDAC6 causes the acetylation and inactivation of heat shock protein 90, and, together with reduced expression of the chaperones HSP70 and GRP78, generates a strong endoplasmic reticulum (ER) stress response. Prolonged intense ER stress signaling causes tumor cell death. Reduced expression of HDACs 1, 2 and 3 causes the levels of programed death ligand 1 (PD-L1) to decline and the expression of Class I MHCA to increase which correlates with elevated immunogenicity of the tumor cells in vivo. This review will specifically focus on the downstream implications that result from autophagic-degradation of HDACs, RAS and protein chaperones.

1. Introduction

Autophagy is an evolutionary conserved process found in single cell yeasts and in mutli-cellular mammals [1,2]. The basic role of autophagy is to recycle cellular components if they are damaged or denatured, or during times of nutrient stress, to maintain homeostasis and cell viability [3]. The double-membrane autophagosome initially forms around the damaged organelles and/or proteins, and the fuses with acidic endosomes to form an autolysosome [4]. The organelles and proteins are subsequently degraded in the autolysosome and the degraded materials returned to the cell for new uses [5].

The regulation of autophagy in mammalian cells can be explained at two levels; one that is relatively simplistic with regard to altered signaling by mammalian target of rapamycin (mTOR) and another that includes mTOR signaling together with the regulation of expression and phosphorylation of multiple proteins who collectively play roles in the regulation of autophagosome formation, autophagosome fusion with endosomes and autolysosome acidification [6–10] (Fig. 1). Simplistically, mTOR and the AMP-dependent protein kinase (AMPK) coordinately regulate the activity of the Unc-51 like autophagy activating kinase (ULK1). Phosphorylation of ULK1 at COOH-terminal sites by mTOR inactivates ULK1 e.g. S757. Phosphorylation of ULK1 at sites closer to the NH2-terminus of the protein by the AMPK activates ULK1 e.g. S317. Furthermore, it should be noted that signaling by the AMPK can itself cause inactivation of mTOR via phosphorylation of Raptor; mTOR activity is generally thought to be maintained by upstream signaling from the PI3K/PTEN/AKT pathway. This dynamic multi-site phosphorylation of ULK1 means a cell can exquisitely control the ability of ULK1 to phosphorylate its key target, ATG13. Phosphorylation of ATG13 at Serine 318 represents the key gate-keeper step for autophagosome formation. Important additional proteins, such as Beclin1, ATG5, ATG16L1 and LC3 (ATG8) also play essential roles in the formation of the double-membrane autophagosome.

Under normal biological circumstances, the autophagosome matures and then fuses with lysosomes that acidify, facilitating the proteolytic degradation of their contents e.g. by cathepsin and calpain proteases [11–14]. This transition process is termed “autophagic flux.” The anti-malarial drug chloroquine prevents the fusion of the

Abbreviations: ERK, extracellular regulated kinase; PI3K, phosphatidyl inositol 3 kinase; AMPK, AMP-dependent protein kinase; mTOR, mammalian target of rapamycin; JAK, Janus Kinase; STAT, Signal Transducers and Activators of Transcription; MAPK, mitogen activated protein kinase; PTEN, phosphatase and tensin homolog on chromosome ten; HDAC, histone deacetylase; NSCLC, non-small cell lung cancer; HCC, hepatocellular carcinoma; RCC, renal cell carcinoma; PDE5, phosphodiesterase 5; PKG, cGMP-dependent protein kinase; ER, endoplasmic reticulum; HSP, heat shock protein; GRP, glucose-regulated protein

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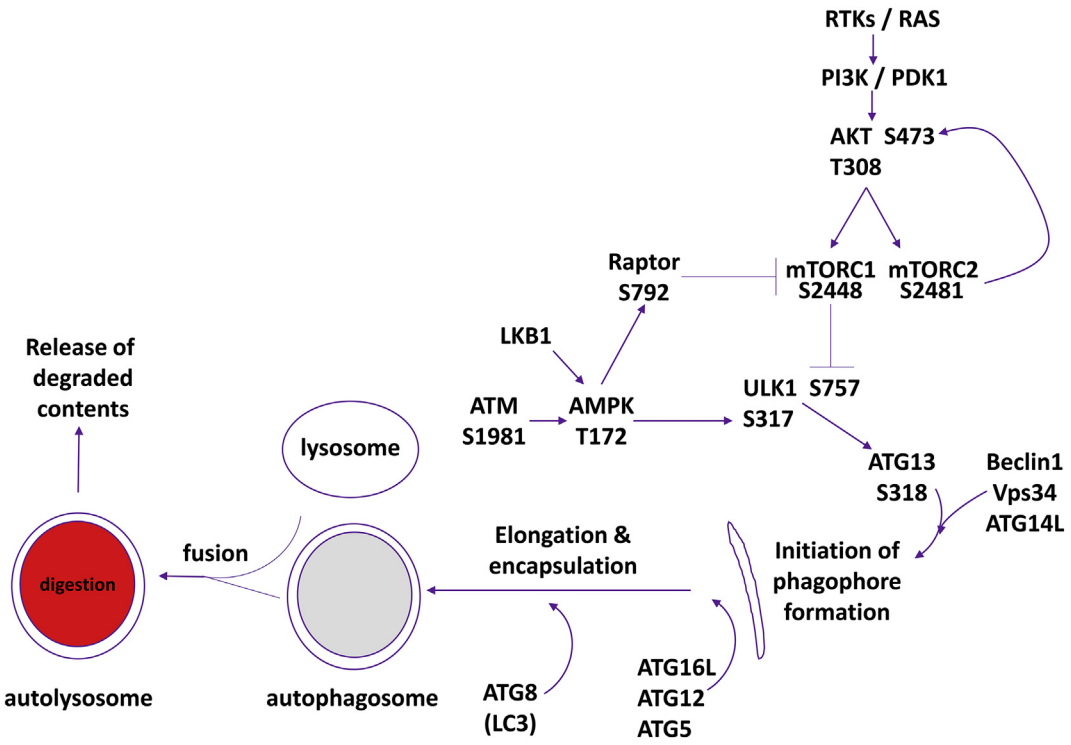

Fig. 1. Proteins and regulatory mechanisms controlling autophagosome formation.

autophagosome with the endosome which causes a build up of autophagosomes within the cell. Depending upon how autophagosome formation was induced, i.e. blocking basal flux versus stimulated flux, the actions of chloroquine may act to enhance or suppress tumor cell killing. This review will focus upon proteins whose expression is regulated by autophagy rather than discussing the complex interplay between autophagy, apoptosis and tumor cell death.

2. Autophagy and the degradation of HDACs, chaperone proteins and mutant K-RAS

There are several recognized 'types' of autophagy including macro-autophagy, chaperone-mediated autophagy and LC3-associated phagocytosis (LAP). Many of the observations that will be discussed in this review require, in addition to macro-autophagy, also LAP to reduce protein expression [15–20].

2.1. HDAC expression and autophagy

In several studies published in 2017 we demonstrated that the combination of the non-small cell lung cancer (NSCLC) therapeutic pemetrexed and the phosphodiesterase (PDE) 5 inhibitor sildenafil reduced the expression of the cytosolic histone deacetylase (HDAC) 6, thereby increasing the acetylation and decreasing the chaperone function of its client, heat shock protein (HSP) 90 [21,22]. Knock down of the AMPKα, ATG5 or Beclin1 prevented HDAC6 degradation proving that HDAC6 destruction required macro-autophagy. We went on to demonstrate in NSCLC and ovarian cancer cells that [pemetrexed + sildenafil] exposure not only reduced HDAC6 levels, but also the expression of many other HDAC proteins (HDACs1-11). Knock down of Beclin1 prevented the drug-induced degradation of the HDAC proteins. The HDAC inhibitor apicidin can suppress expression of HDAC8 in squamous carcinoma cells [23]. HDACs, simplistically as their name suggests, regulate the acetylation of histones, with histones regulating the condensation status of DNA i.e. and the regulation of transcription [24,25]. Some HDACs, such as HDAC6, are cytoplasmic, though the majority are nuclear [26]. Acetylation of histones results in the loosening of histone – DNA interactions and in theory, this permits relaxation of the DNA which facilitates transcription factor association with promoter elements leading to increased transcription. However, in practice, treatment of cells with HDAC inhibitors both enhances and suppresses gene function in equal measure [27,28].

As single agents, we have also observed that HDAC inhibitors at physiologic concentrations can rapidly enhance autophagosome formation. There are several potential mechanisms by which HDAC inhibitors can enhance autophagosome formation. The most obvious is that via inhibition of HDAC6, HSP90 and other chaperones become acetylated and inactive, leading to greater levels of denatured proteins and enhanced ER stress signaling. In addition, HSP90 is also a key chaperone for growth factor receptors and signaling by RAF-1 and AKT. Loss of HSP90 function is associated with inactivation of AKT, with a resultant inactivation of mTOR and enhanced autophagosome formation. Although no data is extant on the acetylation of autophagy-regulatory proteins directly, it is likely that altered acetylation of "ATG" proteins will facilitate protein complex associations and autophagosome formation. In our work, we have yet to observe a drug combination that did not in parallel increase autophagosome formation and also reduce HDAC protein levels, e.g. the combination of the multi-kinase inhibitor pazopanib with HDAC inhibitors in melanoma and in sarcoma cells [29,30].

From a simple cell signaling perspective, the use of multi-kinase inhibitors such as sorafenib and pazopanib, or drugs such as sildenafil that activate protein kinase G and generate nitric oxide will as single agents alter the activity of multiple MAP kinase pathways as well as of the PI3K/AKT pathway which in turn will change the levels of transcription factor phosphorylation and thus the regulation of transcription. The discovery that autophagy as a secondary event can reduce HDAC expression adds a new layer of complexity to these processes. Our current working hypothesis is that if a drug or drug combination causes significant levels of autophagosome formation alongside causing autophagic flux with protein degradation, then the levels of at least some of the nuclear HDAC proteins will be reduced alongside cytosolic

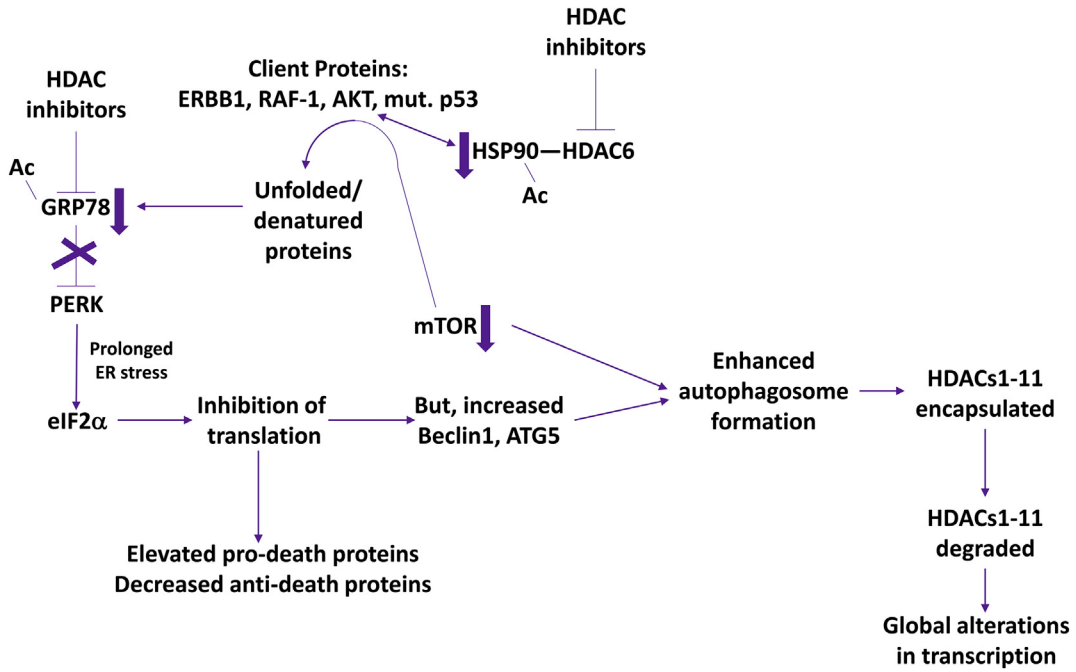

Fig. 2. Proteins and regulatory mechanisms by which autophagy is triggered by HDAC inhibitors and how autophagy reduces HDAC protein expression.

HDAC6 (Fig. 2). And hence, that the impact upon transcription of any drug combination that enhances autophagosome formation will be the complex sum total of altered activity within signal transduction pathways that regulate transcription factor phosphorylation and with reduced expression of HDAC proteins that regulate DNA condensation and promoter availability. What became clear from our in vivo studies using drug combinations that stimulate autophagy and reduce the expression of HDAC proteins was that fourteen days after cessation of drug treatment, cells still maintained a lower expression of multiple HDAC proteins, as though the autophagy-dependent HDAC down-regulation profoundly altered the biology of the surviving tumor cells [31].

2.2. Chaperone proteins and autophagy

The ATPase activities and protein-protein interactions of chaperones are regulated in a fine-tuned fashion via phosphorylation, acetylation and methylation [32–35]. For example, enhanced acetylation of HSP90, caused by degradation of HDAC6 or use of an HDAC inhibitor, results in a lower chaperone ATPase activity and a reduced ability of HSP90 to associate with client proteins such as ERBB1 (the epidermal growth factor receptor), PDGFRβ, (platelet derived growth factor receptor beta), RAF-1 and B-RAF [36,37]. Chaperone expression itself can be controlled by ubiquitination, sumoylation and transcription. In 2016, we presented evidence that the combination of the NSCLC therapeutic pemetrexed with the hepatocellular carcinoma (HCC) / renal cell carcinoma (RCC) therapeutic sorafenib rapidly reduced the expression of multiple HSP70 family and HSP90 family chaperone proteins which was associated with lower expression of the oncogene c-MYC and with enhanced endoplasmic reticulum (ER) stress signaling, including phosphorylated inactivated eIF2α, which were collectively causal for drug-induced tumor cell death [38]. Similar findings regarding autophagy and chaperone expression were made using several additional drug combinations, e.g. the sarcoma therapeutic pazopanib combined with HDAC inhibitors, all of which significantly enhanced autophagosome formation, and that via autophagy, caused chaperone degradation [29,30].

Hence, in a fashion similar to that proposed for HDAC expression, signaling and transcription, the ability of autophagy to control the levels of chaperone proteins also adds a new multi-factorial concept to the mechanisms of drug action. A drug combination may inactivate receptors or signaling intermediaries via direct on-target effects whilst in parallel enhancing autophagosome formation and autophagic flux. The increased flux and resultant degradation of chaperone proteins will result in two broad outcomes. First, reduced expression of the chaperone GRP78 leads to enhanced eIF2α phosphorylation; eIF2α phosphorylation reduces translation for the majority of mRNAs, though for some transcripts such as the autophagy regulatory proteins Beclin1 and ATG5, their protein levels are increased [39]. Second, simultaneous lowering of HSP90 and HSP70 expression is highly detrimental to the stability and function of multiple protein kinases whose functions, broadly, are to maintain viability. Work from a decade ago using geldanamycin family drugs, which inhibit the ATPase activity of HSP90, demonstrated that as the cell lost HSP90 function, it compensated for this by increasing the expression of HSP70 [40]. Molecular knock down of HSP70 in the presence of a geldanamycin drug demonstrated a synthetic lethality for tumor cells [41]. Thus, as a secondary effect, autophagic-dependent reductions in chaperone expression will tend to increase ER stress signaling which reduces the levels of protective proteins with short half-lives such as MCL-1 and that via Beclin/ATG5 causes more autophagy whilst by promoting kinase denaturation in parallel will block any potential compensatory activation of survival signaling pathways [42] (Fig. 2).

2.3. RAS and autophagy

During the project that defined the molecular mechanisms by which pemetrexed and sildenafil interacted to kill NSCLC cells, we generated in vivo NSCLC tumors that were resistant to a standard of care therapeutic, the irreversible ERBB1/2/4 inhibitor afatinib [43]. The resistant cells had reduced the expression of both ERBB1, ERBB4 and PTEN [44]. Using molecular tools, we demonstrated that enhanced signaling by SRC/ERBB3, c-MET and c-KIT played key roles in the afatinib resistance process. As a comparator to afatinib, we utilized the recent FDA approved irreversible ERBB1/2/4 inhibitor neratinib. To our surprise, afatinib-resistant NSCLC cells remained and in fact were more sensitive to neratinib than the control cells [45]. These findings prompted us to perform additional analyses to understand why

neratinib could kill the afatinib-resistant cells. The first indication of the different biology of neratinib compared to afatinib was that neratinib caused the rapid degradation of ERBB1, ERBB3 and ERBB4; the cells expressed negligible amounts of ERBB2 (also called HER2). As a negative control, we also examined the levels of c-MET and c-KIT, and much to our surprise, these receptors were also degraded after neratinib exposure, n.b. neratinib does not alter the tyrosine phosphorylation of either c-MET or c-KIT [454]. For ERBB1, the degradative process required ubiquitination and a subsequent autophagic digestion whereas for c-MET the degradative process relied solely on autophagy. It has been postulated for many years that receptors and intracellular signal transducers localize in the plasma membrane in quaternary structures, which is proposed to facilitate coordinated fine-tuned cell signaling [46]. Thus, we postulated, if we are down-regulating multiple growth factor receptors, are we also down-regulating small GTP binding proteins located on the plasma membrane's inner leaflet, i.e. RAS proteins?

Approximately one third of human tumors express a mutant RAS protein, predominantly mutant K-RAS, with at least another third of wild type RAS proteins being constitutively GTP bound due to upstream signaling by growth factor receptors [47,48]. Our initial studies examining K-RAS expression first had to validate our K-RAS antibody, as the majority of commercially available antibodies raised against this protein are non-specific [49]. Using our validated antibody, neratinib, and particularly neratinib when combined with an HDAC inhibitor reduced the expression of wild type and mutant K-RAS proteins [45]. Microscopic examination at 60× magnification of the drug-treated cells stained with the anti-K-RAS antibody revealed the presence of intracellular vesicles that were liminal to the plasma membrane. The intracellular vesicles containing K-RAS co-stained for phosphorylated ATG13 S318 and for Beclin1, i.e. the vesicular K-RAS was localized in autophagosomes.

Using plasmids to express GFP- and RFP-tagged forms of K-RAS V12 we demonstrated that neratinib rapidly caused the formation of intracellular vesicles staining intensely both for GFP + and for RFP + [50] (Fig. 3). In an autophagosome both GFP and RFP fluoresce whereas in the acidic endosome GFP fluorescence is quenched and only RFP staining is observed. In neratinib treated cells, for the following 4 h after exposure the GFP + and RFP + vesicles colocalized, however, 8 h after exposure, vesicles staining for only RFP were observed. Furthermore, the amount of RFP fluorescence was reduced compared to control treated cells. Thus, neratinib was causing mutant K-RAS to initially localize in autophagosomes and that autophagic flux was occurring which was associated with an overall reduction in the amount of RFP fluorescence in the cells. We then performed additional confirmatory analyses using a different methodology to confirm our initial findings. Non-transformed cells were transfected to stably co-express GFP tagged K-RAS V12 and with Cherry-CAAX that acts as a general

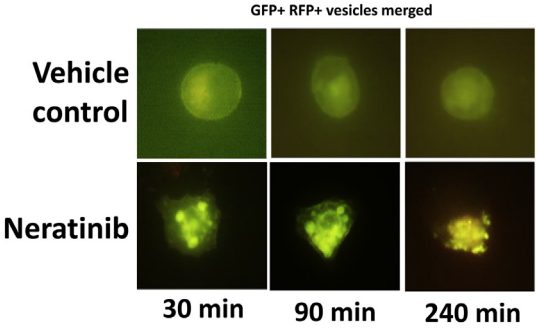

Fig. 3. Neratinib causes the rapid intracellular vesicularization of GFP-K-RAS V12 and RFP-K-RAS V12. PANC1 pancreatic cancer cells were co-transfected with plasmids to express GFP-K-RAS V12 and RFP-K-RAS V12. Twenty-four hours later, cells were treated with vehicle control or with neratinib (50 nM) for 30, 90- and 240-min. Live cells were imaged at each time-point at ×60 magnification.

endomembrane marker. Neratinib-induced the mislocalization of the GFP tagged K-RAS V12 from the plasma membrane to the endosomal membranes where it co-localized with the Cherry-CAAX protein [51,52]. Twenty-four and 48 h after neratinib exposure, the fluorescence levels of Cherry-CAAX were unchanged whereas the expression of GFP tagged K-RAS V12 was abolished. Thus, neratinib can relocate RAS proteins from the plasma membrane into intracellular locations where the protein can be subsequently degraded.

Other laboratories have also observed that RAS protein levels can be reduced via processes and agents that enhance autophagosome formation. For example, poly-ubiquitination of RAS can facilitate its autophagic degradation [53]. Others have shown that Aplasia Ras homolog member I (ARHI), a tumor suppressor, regulated RAS protein levels in glioma cells via autophagy [54]. RAS-inhibitory drugs and the calcium channel blocker and histamine H1 blocker Flunarizine also have been shown to down-regulate the expression of RAS proteins via autophagy [55,56].

2.4. Neratinib and MAP4K inhibition

Our studies using neratinib then diverged with several projects in different malignancies examining the molecular interactions between neratinib and HDAC inhibitors. Uveal melanoma, unlike cutaneous melanoma, does not exhibit mutations in RAS proteins or B-RAF. Instead, uveal melanoma cells with a ~90% penetrance express mutated GTPase-inactive forms of the hetero-trimeric GTP binding proteins, either $G\alpha_q$ or $G\alpha_{11}$ [57,58]. Neratinib as a single agent and to a greater extent when combined with an HDAC inhibitor reduced the expression of $G\alpha_q$ or $G\alpha_{11}$ via autophagy [52,59]. Thus, it appears that neratinib is capable of downregulating expression of plasma membrane GTP-binding proteins that do not normally associate with its recognized receptor tyrosine kinase targets of ERBB1/2/4. As mentioned previously, afatinib-resistant cells expressed lower levels of ERBB1 and ERBB4 yet were more readily killed by neratinib than control cells. Collectively, these findings suggest that neratinib has additional targets beyond ERBB1/2/4 to cause degradation.

Studies by Davis et al. in 2011 assessed the inhibitory properties of ~40 kinase inhibitors against ~400 protein kinases using wet-work and chemical biology approaches [60]. This manuscript revealed that whilst afatinib exhibited a restricted specificity for inhibiting only ERBB1/2/4, neratinib could inhibit both ERBB1/2/4 as well as multiple MAP4K proteins, n.b. serine / threonine kinases, in the low nanomolar range. Serine / threonine kinases inhibited by neratinib below a 100 nM IC50 include MST2, MST3, MST4, MAP4K1, MAP4K3, MAP4K5, GCN2 and MAP3K4. The kinases MST1/2/3/4 as well as MAP4K1/3/5 play important roles in the regulation of interactions between the plasma membrane and the cytoskeleton with the Golgi, as well as controlling activity within the Hippo pathway [61–63] (Fig. 4). Thus, in hematopoietic cancer cells that do not express receptors such as ERBB1/2/4, neratinib was still competent at reducing the expression of K-RAS, and albeit weakly, neratinib and HDAC inhibitors interacted to kill blood cancer cells, i.e. modulation of MAP4K function by neratinib is more important in the downregulation of plasma membrane GTP binding proteins that inhibition of ERBB1/2/4 [52].

2.5. Neratinib, the Hippo pathway and LAP

The Hippo signaling pathway controls organ size, though in cancer it promotes tumor cell growth and invasion, and resistance to apoptosis [63]. The serine/threonine kinase MST1 as well as other MAP4Ks act to enhance the activity of LATS1/2. LATS1/2 phosphorylate the co-transcription factors YAP and TAZ. Phosphorylated YAP and TAZ exit the nucleus and are ubiquitinated and degraded in the cytoplasm. Hence, inhibition of upstream MAP4Ks would simplistically imply that the downstream Hippo pathway transcriptional effectors YAP and TAZ would be in their unphosphorylated states, thus, located inside the

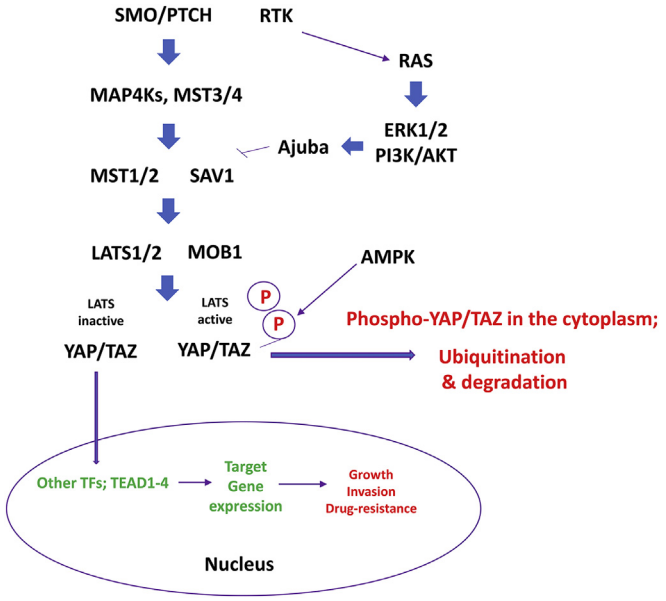

Fig. 4. Regulation of the Hippo Pathway by MAP4Ks.

nucleus and promoting tumor growth and survival. This could limit the anti-tumor efficacy of neratinib. However, we discovered that neratinib actually *increased* the phosphorylation of Hippo pathway regulatory kinases, e.g. LATS1/2, i.e. enhancing their activity, and this in turn elevated the phosphorylation of the LATS1/2 downstream effectors YAP and TAZ; this caused YAP and TAZ to exit the nucleus [52] (Fig. 4). At present, we do not know the precise molecular mechanisms by which neratinib, via MAP4K inhibition, or by causing a compensatory activation of other MAP4K protein kinases, regulates the Hippo pathway, or the processes that are initially involved to regulate membrane trafficking of GTP binding proteins such as K-RAS or Gα proteins. One additional finding from this work, however, that may point the way to further understanding how neratinib regulates the Hippo pathway is that in the absence of the LC3-associated phagocytosis (LAP) and autophagy regulatory protein Rubicon, neratinib causes YAP and TAZ dephosphorylation, suggesting that plasma membrane vesicularization and a protein degradation step is essential for neratinib to suppress the activities of YAP and TAZ [52,61].

One mechanism by which plasma membrane proteins can be internalized and then digested via autophagy is LAP [64,65]. LC3-associated phagocytosis has been extensively studied in the field of immunology, particularly with regard to host – microbial pathogen engulfment and subsequent antigen presentation. More recently, LAP has been shown to play a key role in other pathologies, for example, digesting beta-amyloid protein in mouse models of Alzheimer's Disease [66]. An essential regulator for LAP is the protein Rubicon [67]. Rubicon promotes LAP whilst at the same time suppressing canonical macro-autophagy. In the absence of Rubicon, as well as of Beclin1 or ATG5, neratinib could not downregulate the expression of growth factor receptors or of GTP binding proteins. And, knock down of Rubicon, Beclin1, ATG5 or ULK1 significantly reduced, but did not abolish, the efficacy of neratinib as a single agent or when combined with HDAC inhibitors [52]. Rubicon-induced LAP/autophagosome formation does not require ULK1/ATG13 phosphorylation yet knock down either of Rubicon or of ULK1 both partially preserved viability arguing that both canonical macro-autophagy and LAP each play distinct roles in facilitating neratinib-induced cell killing.

2.6. Neratinib, autophagy, HDACs and checkpoint immunotherapy

The use of checkpoint inhibitory immunotherapy antibodies has become a standard of care approach to treat many tumor types [68–73]. The antibodies for therapeutic use targeted PD-L1, PD-1 and CTLA4 [74–76]. Many types of tumor, e.g. breast and colon cancers, are considered to be “cold” with respect to immunotherapy responses. Antibodies to inhibit other targets are in various stages of development. Multiple HDACs have been implicated in regulating expression of PD-L1, PD-1 and CTLA4, proteins whose levels are used to predict for a response to checkpoint inhibitory immunotherapy antibodies [77–79].

In the majority of cell types tested with our drug combinations, the expression of HDACs1/2/3 and HDAC6 were routinely suppressed via autophagic degradation [21,22,29,30,31,45,50,52,59]. This correlated with tumor cells rapidly reducing the expression of programed death ligand 1 (PD-L1), ornithine decarboxylase (ODC) and Indoleamine 2,3-dioxygenase 1 (IDO-1) and increasing the expression of Class I major histocompatibility protein (MHC) A. Little change in the very low levels of tumor cell PD-1 were observed. In multiple studies we then used transient siRNA knockdown approaches to knock down the expression of individual HDAC proteins or combinations of HDAC proteins to link drug-induced effects on HDAC levels and protein expression with molecular analyses. In a cell-type dependent fashion we were able to associate alterations in the expression of HDACs1, 2, 3 and 10 to changes in the expression of the immunotherapy regulatory proteins, with PD-L1 levels declining and MHCA expression increasing.

Multiple laboratories have been focusing on the use of HDAC inhibitors as a means to enhancing the efficacy of immunotherapy, including the use of those ‘specific’ for HDAC6 [77–79]. In our work, knockdown of HDAC6 did not significantly modulate either PD-L1 or MHCA expression. In our studies combining the NSCLC drug pemetrexed with the PDE5 inhibitor sildenafil, as previously mentioned, we observed the autophagy-dependent degradation of HDAC proteins, and we went on to demonstrate that these changes in HDAC expression were causal in the elevated expression of MHCA and decreased expression of PD-L1, i.e. the pemetrexed plus sildenafil combination acted as a de facto HDAC inhibitor. Furthermore, in vivo, the pemetrexed plus sildenafil drug combination enhanced the lethality of an anti-PD1 antibody against NSCLC cells. These findings open up the possibility for established drug combinations that are known to induce autophagic flux to be rationally used as immunotherapy neoadjuvants.

2.7. Autophagy and drug concentrations

From a translational cancer research perspective, it is also important to discuss the use of therapeutic agents when measuring and assessing cellular responses such as viability, autophagosome formation and the degradation of HDACs and chaperones. For newly created agents undergoing pre-clinical testing in vitro, cell-based studies first need to define the IC50 for the inhibition of the target enzyme. For drugs that are FDA approved, however, before any laboratory-based study is performed, it is vital for investigators to determine from the literature the safe maximal plasma concentration (C_{max}) and the area under the curve (AUC) showing the plasma drug concentration over time. Usually, information is also provided by a drug company as to how much of their drug is protein bound, probably inactive, in the plasma. All of this information can be used to empirically judge at what concentration a drug should be used for cell-based studies, combined with other agents. For example, the plasma C_{max} of sorafenib tosylate following a 400 mg ingestion is ~13 μM. However, sorafenib tosylate is > 90% protein bound in the plasma. Thus, for meaningful in vitro studies, the maximum drug concentration for cells growing in 10% (*v*/v) serum cannot realistically be above 2 μM [80,81]. The reasoning for this approach is that studies using a drug at a physiologic concentration may yield different biological information on autophagy and cell signaling processes versus studies using concentrations an order or two orders above the safely achievable plasma drug level in a patient.

3. Discussion

The ability of anti-cancer therapies to regulate signal transduction pathways was first examined in the mid-1990s, e.g. [82]. One of the first observations made, when physiologic concentrations of drugs or doses of ionizing radiation were used, was that drugs/radiation in parallel to killing tumor cells also activated signal transduction pathways which would be predicted to promote viability, growth and invasion [83,84]. From these findings resulted additional papers which provided evidence for, first, a greater understanding of dynamic signaling fluidity between pathways, and, second, revealed complex processes by which tumor cells attempt to maintain their survival. For example, the dynamic interplay between different MAP kinase pro-survival versus pro-death pathways via altered GTP binding protein signaling was shown to control tumor cell radiosensitivity downstream of radiation induced ERBB1 activation [85]. Many of the early descriptive studies examining the impact of growth factors on pathways only explored changes in signaling within the first 60–90 min of exposure. Radiobiology studies subsequently demonstrated that the ability of a single dose of ionizing radiation would cause two subsequent waves of elevated signaling, with the latter occurring at 120–180 min, and was caused by autocrine feedback signaling, e.g. by TGFα [86–90]. The ability of radiation to activate an ERBB1-TGFα autocrine loop was dependent on the initial signaling processes induced by the radiation itself, i.e. activation of ERK1/2. Of perhaps greater concern for patients undergoing radiotherapy was that irradiated metastatic prostate cancer tumors several hours post-radiotherapy evolved TGFα into the plasma of patients. This may explain why the irradiation of one metastatic tumor can sometimes correlate with the enhanced growth of unirradiated tumors, i.e. plasma TGFα acting on distant tumors to promote growth. Studies using multi-target inhibitors of PI3K, as well as using molecular tools blocking AKT signaling, demonstrated that this pathway represented a more potent cell survival signal than those emanating from the ERK1/2 MAP kinase pathway [84–88]. Downstream of PI3K/AKT signaling is mTOR. Studies in the early 2000s linked PI3K signaling to the regulation of cell metabolism in large part via mTOR, and other studies linked mTOR to the regulation of autophagosome formation [91,92].

The role of mTOR and autophagy with respect to cancer therapeutics, in the broadest sense, is often somewhat confusing to “the uninitiated.” This is because many publications, and even review articles, do not clear demarcate between basal and stimulated levels of autophagy, consider the duration of autophagic stimuli or whether autophagic flux actually occurs. It is known for mammary carcinomas that loss of Beclin1 expression facilitates the tumorigenic process, and that for estrogen resistance to develop the cell must then evolve to re-express Beclin1 [93,94]. Thus, drug combinations which include autophagosome formation as part of their killing mechanism more effectively kill estrogen-independent mammary carcinoma cells. Chloroquine (and its derivatives) prevent the fusion of autophagosomes with endosomes, resulting in an interrupted flux and a build-up of autophagosomes [95,96]. Many studies have shown that inhibition of basal autophagic flux by chloroquine can be detrimental to tumor cell viability, and multiple clinical trials are presently determining the safety and efficacy of this family of drugs [97–99]. The combination of chloroquine with standard of care therapeutic agents has also resulted in a complex milieu of findings. Some data sets argue that chloroquine enhances chemotherapy-induced killing and others that it suppresses drug-induced cell death; such divergent data may be explained based on the drug concentrations used [100,101].

With respect to our own findings using multi-kinase inhibitors in combination with other modalities is the general observation that the kinase inhibitor enhances autophagosome formation, which is then synergistically enhanced by other agents such as HDAC inhibitors or PDE5 inhibitors. In one instance, combining sorafenib with vorinostat in HCC cells, we observed autophagosome formation protect cells against death receptor signaling; a regulatory mechanism more deeply researched by seminal studies from the Thorburn laboratory [102–107]. In all other instances, including the sorafenib / vorinostat drug combination in other tumor types, our observation has been that molecular knock down of ULK1, Beclin1 or ATG5 significantly reduces tumor cell killing [108–113]. Using this over-arching concept, we have subsequently translated many multi-kinase inhibitors plus autophagy modulatory second agents into the clinic (NCT02349867, NCT03919292, NCT01450384, NCT02795819, NCT02624700, NCT02466802, NCT01075113, NCT01817751).

4. Conclusions

The role of autophagy in controlling the response to combinations of mutli-kinase inhibitors with HDAC inhibitors or PDE5 inhibitors is complex and multi-factorial. The findings that autophagosome formation followed by autophagic flux can reduce the expression of HDAC proteins as well as chaperone proteins adds two additional layers of complexity in understanding how autophagy regulates cell fate. These findings also imply that many of the chemical biology in silico studies that attempt to model cellular responses to chemotherapeutics are lacking several key algorithms regarding autophagic regulation of HDAC and chaperone expression. In terms of future directions for the development of multi-kinase inhibitors, our findings over several years, and as outlined in this review, argue that these agents can rapidly increase endoplasmic reticulum stress signaling which both acts to enhance autophagosome formation and decrease protein translation. Therefore, the rational combination of additional agents that will further enhance autophagosome formation, e.g. the diabetes/AMPK activating drug metformin, or endoplasmic reticulum stress signaling, e.g. the non-steroidal anti-inflammatory drug celecoxib, would be predicted to further enhance the lethality of the multi-kinase inhibitor. As many of these second agents are relatively inexpensive, this could also change the cost-benefit analyses for the use of multi-kinase inhibitors; inhibitors which are often not available in many nations due to their cost. As drugs such as sorafenib lose patent protection and as generic drugs are considerably cheaper, this collectively argues new drug combination regimens will become available for many additional nations.

Declaration of Competing Interest

None.

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