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Autophagy, a major adaptation pathway shaping cancer cell death and anticancer immunity responses following photodynamic therapy

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Abbreviations: CD – cluster of differentiation; CMA – chaperone-mediated autophagy; CRT – calreticulin; DAMP – damage-associated molecular pattern; Ecto – surface exposed or surface tethered; ER – endoplasmic reticulum; HSP – heat-shock protein; Hyp - hypericin; ICD – immunogenic cell death; IFN – interferon; IL – interleukin; PDT – photodynamic therapy; Phox – photo-oxidative; ROS – reactive oxygen species;

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Abstract

Autophagy is a major catabolic pathway in a eukaryotic cell, employed for cellular self-degradation of obsolete or damaged cytoplasmic components serving as a major quality control and recycling mechanism that supports cell survival. Autophagy is fundamentally a cytoprotective and pro-survival process yet in general, it has become clear through a number of studies that autophagy has an exceedingly contextual role in cancer biology; conditional on which phase, location or type of oncogenic trigger and/or therapy is under consideration, the role of autophagy could end up fluctuating from pro- to anti-tumourigenic. Numerous studies have revealed that, contingent on the photosensitiser under consideration, autophagy triggered by PDT either adds to therapy resistance (by suppressing cell death) or vulnerability (by enabling autophagic cell death). Beyond cell death regulation, cancer cell-associated autophagy also supports resistance against PDT by reducing anticancer immune effector mechanisms. In this review, we have concisely described the state-of-the-art and the prevailing gap-in-knowledge vis-à-vis the role of PDT-triggered autophagy in cancer therapy resistance or susceptibility.

Introduction

Autophagy is possibly the major catabolic pathway in eukaryotic cells, which is exploited for both cellular self-degradation, to support protein and organellar homeostasis and quality control mechanisms, as well as recycling of biomolecular resources, to maintain energy homeostasis and cell survival .[1, 2] Although autophagy is constitutively present at basal thresholds in a cell yet, it is primarily induced under stressful circumstances (e.g. organelle-associated stress or nutrient deficiency).[2, 3] Autophagy helps in replenishing nutrients through degradation of prevailing damaged or stressed subcellular components thereby abrogating cellular stress.[1, 4] Similarly, if physiochemical stress is applied on a cell that damages specific cellular/subcellular locations, then the resulting signalling pathways and damage-related by-products may compromise cellular growth competence.[2, 5] In such cases, stimulation of autophagy helps eliminate the damaged cytoplasmic components, thereby regulating the stress signalling and extending cellular persistence. For example, the autophagic removal of dysfunctional or oxidatively damaged proteins and mitochondria can prevent the accrual of aggregation-prone proteins and the undesired generation or amplification of toxic reactive oxygen species (ROS) by mitochondria.[2, 6, 7] Of note, organelle localized stress or loss of proteostasis may stimulate more specific forms of autophagy, e.g. reticulophagy (targeted towards the damaged endoplasmic reticulum/ER), mitophagy (targeting the damaged mitochondria), pexophagy (targeting the damaged peroxisomes) or aggrephagy (the specific removal of protein aggregates or aggresomes).[3, 8, 9] In general, as apparent, autophagy is fundamentally a cytoprotective process supporting cell survival.[10]

Maximum variety of autophagic pathways have been confirmed to exist in yeast;[3] mainly consisting of macroautophagy, piecemeal microautophagy of the nucleus, chaperone-mediated autophagy (CMA), cytoplasm-to-vacuole targeting and microautophagy.[1] In mammals however, the presence of mainly macroautophagy (hereafter referred to as simply autophagy, unless otherwise mentioned), CMA and microautophagy has been established[1, 4] while the presence of other autophagic pathways remains unknown. CMA and microautophagy tend to be highly target-specific degradative processes, even if the damage is extensive and not just limited to their respective targets.[3] Perhaps in molecular terms, autophagy demands a far more complex and varied set of signalling components than CMA or microautophagy – an inference that needs comprehensive investigation.

Cytoplasmic biomolecular constituents and entire organelles are sequestered through autophagy, as cargo, into double-membraned vesicles termed as autophagosomes.[1, 2] These, autophagosomes subsequently fuse with the lysosomes, where the autophagosome-cargo is degraded (through the agency of various lysosomal catabolic enzymes) and recycled.[3] Thus during autophagy, the autophagic cargo is indirectly merged with the lysosomes through the support of autophagosomes.[2, 3] However,

microautophagy involves lysosome-based direct engulfment of cytoplasmic objects *via* lysosome-membrane based invagination, protrusion/septation and vesicle scission into the lumen.[11] On the other hand, CMA is a selective kind of autophagy where the cytosolic chaperone heat shock cognate 70 (Hsc70) binds particular damaged proteins comprising a motif matching or analogous to the pentapeptide KFERQ and helps in taking these specific proteins through the lysosomal membranes and into the lysosomal lumen for degradation.[12] This process of protein translocation is aided by the CMA-essential receptor, lysosome-associated membrane protein 2A (LAMP2A).[12] Of note, autophagy and CMA (to a comparatively restricted degree) have been found to play significant roles in cancer metabolic "microevolution", cancer progression and cancer therapy response.[2]

Since, many excellent reviews have recently described the molecular pathways facilitating mammalian autophagy,[2, 13-16] we are avoiding a redundant discussion concerning the same in this review. We would like to refer the readers to these comprehensive reviews for further reading.[2, 13-16] In this review, we will be focusing on the functional and therapeutic implications of autophagy induced by photodynamic therapy (PDT) in cancer. We will summarize the contemporary state-of-the-art and the prevailing gap-in-knowledge concerning PDT-induced autophagy in cancer susceptibility and resistance to therapy.

The capacity of PDT to prompt cell death depends on a systematic course of actions. This therapeutic process starts with the accumulation of a photosensitive "drug" in the cancer cells followed by its stimulation (in the presence of oxygen) *via* light irradiation (of appropriate wavelength which matches to the excitation spectra of that particular photosensitive "drug"); all of which cooperatively brings about the formation of ROS within the cells.[17-19] This ROS-based "photo-oxidative (phox) stress" has the capability to cause cancer cell death.[8, 17] On several levels PDT as a therapy is very different from typical systemic chemotherapeutics (e.g. anthracyclines, various DNA-intercalating or alkylating chemotherapeutics, microtubule-targeting drugs), loco-regionally applied chemotherapeutics (e.g. melphalan) and physical anticancer therapies (e.g. radiotherapy, high hydrostatic pressure).[8, 20, 21] For example, the distinctive dual-component therapeutic approach of PDT (i.e. photosensitive "drug" stimulated following excitation through light irradiation) such that each of these components is innocuous alone but harmful when combined, provides a therapeutic archetype that is mechanistically exciting.[17] This status quo is made more remarkable by the fact that PDT can be used to spawn subcellular organelle-specific stress; since every photosensitive "drug" has a distinctive subcellular localization profile either limited to a particular organelle or covering several different organelles e.g. Hypericin can localize mainly to the ER[22] while 5-aminolevulenic acid (5-ALA) can localize mainly to the mitochondria (see Table 1). This is especially fascinating when it comes to autophagy studies because of autophagy's tendency to "chase" damaged organelles for degradation and recycling.

Thus, the exceptional nature of PDT allows higher tractability and advanced insights both in terms of clinical application and preclinical/biochemical examination. These properties are remarkable when studying the role of autophagy in anticancer therapy response.

Autophagy in Cancer Progression and General Therapy Response: A bird's eye view

Cancer cell-autonomous roles of Autophagy

In cancer, there are three temporally discrete levels where autophagy (primarily macroautophagy) plays vital (contextually intersecting or conflicting) roles i.e. initial stages of carcinogenesis before development of clinically-relevant tumour, tumourigenesis before anticancer therapy and relapsed carcinogenesis after anticancer therapy.[2, 16] The role of autophagy during initial steps of carcinogenesis and in tumours before anticancer therapy is chiefly moulded by the mutational, oncogenic and microevolutionary landscape of the cancer cells, cancer cell-stromal cell-immune cell interfaces and metabolic stress related with tumour microenvironment.[2, 16, 23] On the other hand, the role of autophagy during and after anticancer therapy is fashioned both by the pre-existing autophagy in an established tumour and the effects of the particular category of therapeutic modality (on cancer cells, stromal cells and/or immune cells).[16] Across all these stages, evidence advocates that autophagy is hijacked by cancer cells as an immensely malleable and vigorous apparatus to either facilitate the initial steps of carcinogenesis or sustain the continual presence and growth of established tumours post-anticancer therapy.[16, 23]

In circumstance of initial phases responsible for carcinogenesis, some studies have connected faulty autophagy (but in some cases, also CMA) to augmented carcinogenesis.[24-26] Mechanistically, this tumour-suppressor purpose of autophagy has been attributed to the vital cell-autonomous properties of autophagy in alleviating damage[27] (caused as a consequence of neoplastic transformation) and preserving cellular integrity under conditions of metabolic stress (brought about due to carcinogenic "injury").[16] Moreover, it has been detected that autophagy may also halt the development of tumourigenesis by encouraging oncogene-induced senescence, a route believed to avert further tumour progression.[28, 29] Important example of the tumour suppressor role of autophagy includes the monoallelic deletion of the *Becn1* (coding for the pro-autophagic protein Beclin 1) in 40-75% cases of human sporadic breast, ovarian, and prostate cancer associating with increased tumour progression.[24] Recently however these findings became the subject of discussion, when these *Becn1* deletions were found to be just passenger deletions associating with the tumour suppressor *Brca1* deletions (since *Brca1* is located in close proximity to *Becn1* on chromosome 17q21).[30] However in this aforementioned

analysis, hepatomas, a tumour type for which allelic loss of *Becn1* or biallelic loss of *Atg5* or *Atg7* have been shown to promote tumour initiation in mice, were excluded.[31] These data indicate that the importance of autophagy in suppressing tumour initiation might be limited to certain tumour types. Moreover the capability of tumour-suppressor genes and oncogenes to engage autophagic pathways may ultimately administer the cargo selection by the autophagosome apparatus, thereby influencing the 'functional plasticity' of autophagy throughout cancer advancement.[2]

In the established tumour however, most studies, using pharmacological autophagy inhibitors as well as genetically-engineered mouse models, have provided compelling evidence indicating that autophagy has a predominant pro-tumourigenic role.[32] Autophagy is usually augmented in advanced tumours, and the maximum levels are often found in defectively oxygenated locales where the necessity for nutrients is amplified along with the requirement to tolerate quite a few forms of metabolic stresses.[2] Thus, clinically-relevant, progressive tumours display an 'autophagy addiction' that is mandatory to conserve their energy equilibrium, through the reprocessing of intracellular constituents.[16, 33] Furthermore, it has been stated that, cancer cell-associated autophagy could also expedite the metastasis of tumour cells by subduing pro-death mechanisms faced during the process of metastatic dissemination e.g. autophagy has been revealed to suppress extracellular matrix (ECM) detachment-induced cell death, (i.e. anoikis), thus raising the likelihood that it could stimulate cancer cell survival in the blood stream following extravasation/ loss of interaction with the ECM [34]. In this situation, autophagy can also be vital for maintaining tumour cell "dormancy" upon extravasation and colonization of a distant location, until a stout cancer cell–ECM contact is re-established at a distant "seeding" location.

Nonetheless, under certain circumstances (comprising treatment with specific anticancer therapeutics), autophagy has been revealed to boost cell death; either by permitting the pro-apoptotic routes or by facilitating "autophagic cell death", a type of cell death mediated (rather than simply escorted) by autophagy.[35-37] It is imperative to note that thus-so-far, these pro-death or 'autophagic cell death' outcomes have been mostly credited to autophagy while CMA has been chiefly revealed to be a pro-survival pathway.[3, 32, 38] The machineries of 'autophagic cell death' in the setting of cancer are still intangible and necessitate additional extensive examination.

Cancer cell non-autonomous roles of Autophagy

The above discussion summarizes the cancer cell-autonomous "jobs" of autophagy in carcinogenesis that results in not only dynamic adaptation to stressful environments but also safeguarding of proteome integrity and energy metabolism. Nevertheless, latest research has evidently indicated that autophagy

also controls a variety of cell-non-autonomous processes.[2, 6, 8, 16, 23, 39] Such cell-non-autonomous (mostly but not always, paracrine) functions of autophagy have extensive influence on the tumour microenvironment and they appear to be controlled by the location, nature of the relevant soluble or cellular mediators, and the intricacy of the tumour cell–stromal cell interactions. For example, knockout of the key autophagy gene *Atg5* in the endothelial cells has been shown to further abnormalise tumour vessel structure and reduce tumour vessel perfusion in mice.[40] Likewise, accumulating research advocates that autophagy may play an imperative role in immunosurveillance of senescent or normal cancer cells.[2] Currently, both cancer cell-associated and immune cell-associated autophagy have been established to be play contextual parts (i.e. either immunoevasive or pro-immunity parts contingent on the situation) in determining the cancer cell-immune cell interactions.[2, 41-43] Finally, a role for autophagy in cancer-associated fibroblasts in assisting energy metabolism and the development of adjacent epithelial cancer cells, has been suggested.[2, 44]

Autophagy plays a contextually dynamic part in cancer instigation and development. Remarkably, this tendency is mirrored by autophagy's role in responses to anticancer therapy; such that therapy responses vary from unchanged or increased, to reduced cancer cell killing upon autophagy blockade.[16, 45, 46] Nonetheless, numerous cancer therapy studies have discovered that in most instances cancer cell-associated autophagy plays a pro-survival role, thus decreasing the cytotoxic effects of anticancer therapeutics. In theory, bearing in mind the deep-rooted role of autophagy in stress alleviation, this is quite believable. Additionally, it has also appeared lately that therapy prompted autophagy in cancer cells has the ability to impact the interface between dying cancer cells and the immune system by regulating the "discharge" of immunostimulatory danger signals or damage-associated molecular patterns (DAMPs).[42, 47] On the whole, contingent on the anticancer therapy under deliberation, the kind of cellular stress they induce, and the autophagic cargo that is selected, cancer cell-associated autophagy can either enhance the cancer immunogenicity or aid in immunoevasion and subjugation of anticancer immunity.[8, 42, 48, 49]

Therefore, on the whole, it is clear that autophagy has a greatly contextual role; contingent on which phase, location or kind of tumourigenesis or therapy intervention is under consideration, the role of autophagy could end up fluctuating from pro- to anti-tumourigenic.

At this instant, an imperative question is, does this overall trend regarding the contextual role of autophagy in anticancer therapy also relates to PDT or is it more resolute? Also, a mechanistically critical question that broad non-PDT research has been principally incapable to answer is: is pro- or anti-tumourigenic role of autophagy after anticancer therapy regulated by the subcellular site of the therapeutic stress and/or by the autophagic cargo degraded? The latter question has great connotation

and in our opinion only PDT is proficient at answering such a question within a therapeutic context. In the subsequent sections, autophagy's role in PDT-based cancer therapy response has been examined and deliberated upon in details.

Autophagy and PDT-induced cell death in cancer: Understanding the double-edged sword

Various *in vitro* studies using several photosensitizers have been published over last six to seven years since the first study on the role of autophagy in PDT appeared.[50] Thus, in order to achieve a broader interpretation of the significance of autophagy for PDT-based therapy response we charted all the studies that have used autophagy blockade approaches to determine the role of this molecular pathway in PDT sensitivity (see Table 1).

Fascinatingly, our survey indicated that, there was approximately same number of occurrences of autophagy playing a pro-survival role as there were occurrences where it played a pro-death role (Table 1 and Figure 1). For example, knocking down specifically the expression of ATG7 in hepatoma 1c1c7 cell lines, increased the cytotoxicity of photosensitisers imparting photodamage to mitochondria and the ER (i.e. BPD or Verteprofin; Figure 2); whereas for those imparting photodamage to the lysosomes (i.e. the chlorine NPe6 and the palladium bacteriopheophorbide WST11) ATG7 knock-down decreased cytotoxicity (without, NPe6/WST11-based PDT inducing lysosomal permeability; Figure 3).[51-53] Interestingly, the latter pro-death effect was specific for ATG7, whereas knock-down of ATG5 did not alter NPe6 or WST11-PDT induced photocytoxicity. In contrast to this observation, ATG5-mediated autophagy has been found to be cytoprotective after photodamage to the ER by Hypericin-based PDT (Hyp-PDT) and after photodamage to the mitochondria by Protoporphyrin IX (PpIX)-based PDT (Figure 2).[38, 42, 48, 54] Moreover, similar to certain other therapies, for PDT as well, CMA was observed to be chiefly playing a pro-survival role even though this has so far been systematically tested for Hyp-PDT only (Figure 2). Whether PDT, in a photosensitiser-specific manner, is able to modulate other autophagy pathways, like microautophagy (i.e. the internalization of cytosolic cargo trough invagination of the membrane) is not known but it will be worth investigating in future studies. It is also worth noting that cytoprotective autophagy induction by certain photosensitisers has been shown to involve rapid downregulation of the mTOR/AKT pathways (which opposes autophagy) (Figure 2), thus suggesting that a differential modulation of crucial upstream regulators of autophagy, may also play a role in deciding whether autophagy triggered by PDT either contributes to therapy resistance or susceptibility (Table 1 and Figure 1).

Of note, it had also been hypothesized formerly on the basis of studies with NPe6-based PDT and WST11-based PDT (both largely affecting the lysosomes) that targeting lysosomes might cause primarily pro-death autophagy induction (or autophagic cell death; Figure 3)[51]; yet more recent studies with different lysosome-targeting photosensitisers (based on chlorophyllin e4/f) showed presence of pro-survival autophagy (Figure 2).[55, 56] Though in case of the latter study and several other studies cited in Table 1, only chemical inhibitors of autophagy (e.g. Bafilomycin A1, 3-MA, CQ or Wortmannin) were employed for autophagy blockade. It is imperative to mention here that chemical inhibitors of autophagy are not very precise and have several off-target effects.[3] Based on this standpoint, these results should be treated with due caution and should eventually be validated by genetic approaches, such as RNAi based knock-down or CRISPR/Cas9 or TALEN based knock-out of autophagy relevant molecules.[3] Notably, wherever conceivable, results acquired in cells exhibiting genetic knock-out of autophagy genes need to be compared with RNAi-based knock-down of that particular molecule since observations in these two systems might sometime differ due to pre-existing compensation mechanisms occurring in the knock-out cells. [42, 48, 57] Last but not least, it might also be desirable in certain contexts to test cell death read-outs in presence of RNAi-based knock-down of more than one autophagy-relevant molecule. This is because sometimes, particular molecules relevant for autophagy may also exhibit the ability to directly modulate cell death e.g. a calpain-mediated ATG5 cleavage product has been shown to directly provoke apoptosis.[58]

It is remarkable that accurate molecular information behind PDT-induced autophagic cell death is presently mysterious. Instead, molecular specifics behind autophagy-based resistance to PDT therapy on the level of cell death have started to appear (Figure 2). Largely using Hyp-PDT system, we have confirmed that PDT-induced oxidative-stress causes substantial build-up of oxidatively damaged proteins[57, 59] and perhaps other modified biomolecules (e.g. peroxidised lipids) at the subcellular location where the photosensitiser originally localised before PDT (since subsequent to PDT, several photosensitisers display the propensity to re-localise).[4] Following this, autophagy principally endeavoured to degrade and recycle the damaged organelle and subcellular locations affected by PDT in a spatiotemporally well-defined fashion.[57, 59] This cytoprotective action of autophagy eventually lead to lowered PDT-associated cell death.[57, 59] For instance, Hypericin mainly localises in the ER and its photoactivation thus causes oxidative ER stress, ER-to-mitochondria transfer of ROS through mitochondria-associated ER membranes and subsequent mitochondrial apoptosis.[4, 22] Successively, autophagy is triggered and firstly attempts to recycle the damaged ER (through reticulophagy) and later attempts to recycle damaged mitochondria (through mitophagy).[57, 59] Beyond, reticulophagy and mitophagy, we also found evidence of induction of aggrephagy after Hyp-PDT.[60] More specifically, we observed that Hyp-PDT induced proteotoxicity stimulates formation of p38(MAPK)-regulated,

p62/NBR1-mediated ubiquitin aggregates which are ultimately removed by aggrephagy.[60] It was observed that this p38(MAPK)-regulated activity was required for counter-acting PDT-induced oxidative stress (through Nrf2-mediated anti-oxidative signalling).[60] This spatiotemporally defined interplay of autophagic pathways consequently culminates into lowering of ER stress and suppression of mitochondrial apoptosis.[57, 59] In this model, we also found that CMA may co-exist with autophagy such that CMA helps in recycling of specific damaged cytosolic proteins and thus enhances cancer cell survival.[57, 59] This cytoprotective role of CMA was found to be additionally prominent when autophagy was genetically inactivated (e.g. in ATG5 knock-out cells).[57, 59] Thus PDT induced autophagy exerts resistance against cell death by recycling the impaired organelles and subcellular entities targeted by PDT-induced oxidative stress.

It is imperative to note though that the "black-and-white" situation described above is not pertinent to all the PDT paradigms. A particularly thought-provoking set-up that may in future aid in resolving the problem about the "switch" that picks between pro-death and pro-survival role of autophagy, is the PDT settings based on Pc4 and 5-ALA. In both these circumstances, opposing studies exist, that support pro-death as well as pro-survival role of autophagy for the same photosensitiser (Table 1). It is essential to note that, this development is not unheard of in general, as comparable conflicting instances have been reported for some other extensively applied anticancer chemotherapeutics like bortezomib,[61] temozolomide[62] and imatinib.[63] There are numerous processes or phenomena that can clarify these contradictory observations. For instance, technically speaking, the nature of cancer cell type used (different "cell culture clones" of cancer cell lines "evolving" spontaneously *in vitro*) and, as mentioned above, the type of autophagy blockade strategy applied can make a difference.[3] On the level of PDT itself, alterations can exist in terms of sub-cellular localization of the photosensitisers – a factor that has been found to be highly susceptible to discrepancies. Last but not least, on the mechanistic level, contextually different outcomes may also happen due to differences in the cross-talk between cell death-associated signalling and autophagy e.g. pro-apoptotic proteins like caspases or calpains can execute the cleavage of autophagy-related proteins (like Beclin 1 or ATG5) that often culminates the latter's gain-of function; similarly autophagy can target pro-apoptotic proteins (like active caspase 8) for degradation;[64] thus, deregulation of one pathway may lead to increased activation of the other.

The final point discussed above may well be pertinent to the case of 5-ALA PDT,[65] where the pro-death role of autophagy might have been detected due to the occurrence of a cancer system (i.e. glioma) with tendency to undergo autophagic cell death.[65] Numerous lines of proofs suggest that glioma system is more susceptible to autophagy-inducing therapies (like temozolomide and imatinib) due to its propensity to undergo autophagic cell death, at least *in vitro*.[62, 63, 66] More explicitly, it has been

witnessed that glioma cells are more likely to react to therapy through excessive autophagy rather than apoptosis, perhaps due to deregulated caspase signalling in these cells[66, 67] Nevertheless, additional studies are obligatory to institute this as a primary purpose for such discrepancies or predisposition.

Last but not least, the precise relationship between organelle-specific stress and functional autophagic result in terms of therapy response is still a perplexing topic that needs additional consideration possibly through the intervention of PDT combined with synthetic biology paradigms. One such encouraging synthetic biology paradigm is genetically-encoded photosensitisers (GEP).[68] Chemical photosensitisers typically do not localise to a specific site and can display discrepancies in cellular localization particularly with respect to certain cancer cell types or the photosensitiser concentration. We envisage that, GEPs[68] directed to particular subcellular organelles may help deciphering the organelle stress-autophagy link in cancer therapy response.

Finally, whether this dichotomous effect exists because these studies involved largely '*in vitro* photosensitisation' rather than '*in vivo* PDT effects' is an enigmatic question that needs further attention in near future.

Cancer cell-associated Autophagy in a Tumour *in vivo*: Still a long way to go for PDT

In vitro cell cultures do not embody the *in vivo* situation where tumour cells constantly "network" with stromal cells or immune cells. As discussed before, tumour stromal cells and immune cells can also have noteworthy influence on autophagy-based anticancer therapy outcome and responsiveness. Therefore, in circumstances where the cancer cell-associated autophagy cross-talks with stromal or immune cells has substantial influence on anticancer therapy, the *in vitro* results (as detailed in Table 1 for various PDT paradigms) may no longer be translatable *in vivo* in their entirety. It is notable that of all the *in vitro* results obtained for several PDT paradigms in the context of autophagy (Table 1) only one study's *in vitro* results have as-yet-been verified *in vivo* (albeit to a limited extent). More specifically, a very recently published study showed that *in vitro*, autophagy plays a cytoprotective and pro-colonogenic role against PpIX-based PDT in $CD133^+$ colorectal cancer stem-like cells (CSCs).[54] The authors went onto confirm these specific observations *in vivo* when they observed that these colorectal CSCs, ablated of autophagy, and treated with PDT *in vitro*, showed highly compromised tumourigenicity when xenografted in immunodeficient mice.[54] A big limitation of this approach is that PDT treatment was not done *in vivo* on a pre-established tumour in an immunocompetent mice thereby bypassing the formation of a competent and preclinically relevant tumour microenvironment. This clearly shows that a more *in vivo* PDT treatment-based analysis is obligatory to entirely illustrate the

tumour-level therapeutic relevance of autophagy in determining cancer resistance or susceptibility to PDT. It would also be interesting to study the role of tumour stromal cells and tumour immune cells relative to autophagy-based modulation of tumour PDT-responsiveness.

Autophagy-mediated suppression of anticancer immune effector mechanisms: An unprecedented emerging paradigm in PDT setting

In current times it has appeared that the surface proteome and/or the secreted proteome of a dying cancer cell consists of specific danger signals or DAMPs that might aid the creation of a fecund interface between the cancer cells and the immune cells, which could diminish therapy resistance and prompt anti-tumourigenic immune reactions.[5, 43] In fact, a cancer cell death sub-routine adept at displaying a surface or secreted proteome "rich" in vital DAMPs has recently been characterized and termed as immunogenic cell death (ICD)[47, 69] – a cell death sub-routine capable of inducing potent anticancer immunity in absence of any adjuvants.[70, 71]

Over the years, various PDT modalities have been shown to induce potent anti-cancer immunity, *in vivo*. Aluminium disulfonated phthalocyanines-based PDT was the first PDT modality delineated to induce host immune system-dependent tumour rejection *in vivo*.[72] Since then, a number of studies have unequivocally established that activation of anticancer immunity is almost mandatory for effective tumour regression following PDT.[8, 73, 74] Moreover, Fotolon-PDT has been shown to induce "abscopal effect"-mediated regression of distant untreated tumours in a human angiosarcoma patient.[75] In fact, a few studies have even suggested that PDT might work better in a clinical setting involving immunocompetent patients rather than immunosuppressed patients[76] – a conjecture that needs to be verified for cancer in prospective clinical trials. PDT has also been frequently reported to induce DAMPs emission from cancer cells especially PDT involving Photofrin,[74, 77] Hypericin,[48, 78] Chlorin e6[79] and recently, Rose Bengal Acetate.[80]

We have recently revealed that Hyp-PDT encourages proficient *bona fide* ICD in numerous cancer model systems *in vitro*, *ex vivo* and *in vivo*.[70, 78] Hyp-PDT triggered ICD is strongly immunostimulatory such that cancer cells experiencing this sub-routine can mediate potent anti-tumour immunity.[42, 70] DAMPs established to be decisive for ICD and anti-tumour immunity (as also induced by Hyp-PDT) include – pre-apoptotically surface exposed calreticulin (ecto-CRT) – an 'eat me' signal, pre/early-apoptotically secreted ATP – a 'find me' and inflammasome activating signal and mid/late-apoptotically released chaperones like HSP70/90 – acting as TLR (toll-like receptor) agonists and 'find me' signals.[70] Of note, Hyp-PDT induced ICD based DC vaccines are currently under testing in pre-clinical trials for

treatment of glioblastoma and metastatic ovarian cancer (Garg et al. unpublished data; Immunotherapy Platform Leuven or ITPL, UZ Leuven, Belgium).

We have detected that following Hyp-PDT, cancer cell-associated autophagy (specifically macroautophagy) has the ability to impact the interface between the dying cancer cells and the immune system by controlling the emission of DAMPs.[42] We found that following Hyp-PDT, autophagy repressed the emanation of ecto-CRT (without affecting ATP secretion) such that autophagy knock-down (achieved through RNAi methodology) triggered roughly two-fold upsurge in ecto-CRT.[42, 48] Furthermore, autophagy knock-down in Hyp-PDT treated cancer cells had functional effects on immunological determinants of anticancer immunity.[81] Autophagy knock-down in cancer cells treated with Hyp-PDT caused improved phenotypic maturation of DCs, better DC-derived IL-6 production and upsurge of DC-mediated clonal expansion of (IFN-γ producing) $CD4^+$/$CD8^+$ T cells.[42] Thus, Hyp-PDT-induced autophagy in cancer cells was observed to play an unprecedented part in therapy resistance by boosting immunoevasion, subduing the cancer cell immunogenicity and abating elicitation of anticancer immune effector mechanisms (Figure 1). Of note, recently it was suggested that also in the paradigm of Rose Bengal Acetate-based PDT, high autophagy tends to suppress ecto-CRT[80] thereby substantiating the proposition that autophagy has a danger signalling-suppressing role in PDT settings.

Fascinatingly, there were some signs that as far as emanation of DAMPs is concerned, autophagy and CMA might play incompatible roles following Hyp-PDT.[42, 48] More specifically, we detected that, fibroblasts deficient in the CMA-essential gene *Lamp2a* are incapable in displaying ecto-CRT after Hyp-PDT.[48] Whether this consequence of CMA on ecto-CRT has any immunological consequences needs further examination. Nevertheless as of now, on the basis of existing data we can assuredly say that autophagy supports resistance against Hyp-PDT by overpowering anticancer immune effector mechanisms.

Conclusion

Accumulating data delineate that although autophagy has a prevalent cytoprotective role in cancer therapy, in some circumstances, autophagy may be turned into a pro-death mechanism. To this end, the distinctive nature of PDT permits sophisticated flexibility and advanced insights with respect to testing and appraisal of autophagy-based cancer therapy responsiveness. Numerous in vitro studies have revealed that, depending on the photosensitiser under consideration, autophagy triggered by PDT either adds to therapy resistance or susceptibility. Therapy susceptibility can be delineated by the capability of PDT to prompt autophagic cell death – a molecularly still largely undefined process that needs further

consideration in future. On the other hand, PDT induced autophagy may increase resistance to cell death by recycling the damaged organelles and subcellular entities targeted by the PDT-induced oxidative stress. Furthermore, cancer cell-associated autophagy also supports resistance against PDT by overwhelming anticancer immune effector mechanisms. From all these annotations it is clear that, it is the nature of the photosensitiser , the cancer cell-type, various autophagy-related signalling and mechanistic factors and the cancer cell-immune cell cross-talk, are all key factors defining the eventual functional role of autophagy in PDT response. Nevertheless this deduction is based on essentially *in vitro* and partially *ex vivo* outcomes and thus there is an immediate necessity to carry out more *in vivo* examination to entirely illustrate the tumour-level therapeutic significance of autophagy in determining cancer resistance or susceptibility to PDT.

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Table 1. A survey of cancer cell-associated autophagy's effect on the therapeutic responsiveness of cancer to treatment with photodynamic therapy (PDT).

Photosensitiser used for PDT	Subcellular localization of the photosensitiser	Experimental Context	Role of Autophagy in Cancer Therapy-response	Experimental Intervention used for Confirming Autophagy's role	Ref.
Macroautophagy and cancer's response to PDT					
5-ALA	Mitochondria	*In vitro* cell cultures	Pro-death	3-MA	[36]
5-ALA	Mitochondria	*In vitro* cell cultures	Pro-survival	ATG7 siRNA	[65]
9-capronyloxytetrakis (methoxyethyl)porphycene	ER Mitochondria	*In vitro* cell cultures	Pro-survival	ATG7 siRNA	[82]
Chlorin NPe6 and Palladium Bacteriopheophorbide WST11	Lysosome	*In vitro* cell cultures	Pro-death	ATG5 knock-out -cells ATG7 knock-out –cells	[51]
Chlorophyllin e4	Lysosome Mitochondria	*In vitro* cell cultures	Pro-survival	3-MA Bafilomycin A1	[55]
Chlorophyllin f	Lysosome Mitochondria	*In vitro* cell cultures	Pro-survival	3-MA	[56]
Graphene quantum dots	Intracytoplasmic vesicles	*In vitro* cell cultures	Pro-death	LC3B knock-out –cells	[83]
Hematoporphyrin	Cellular Membranes	*In vitro* cell cultures	No effect	3-MA	[84]
Hypericin	ER	*In vitro* cell cultures and *ex vivo* immune cell co-cultures	Pro-survival and Suppresses 'eat me' signal ecto-CRT and	3-MA Bafilomycin A1 ATG5/p62 siRNA ATG5 shRNA ATG5 knock-out -cells	[42, 57, 59, 85, 86]

			anti-cancer immune effector mechanisms	p62 knock-out – cells	
Mesochlorin	Mitochondria	*In vitro* cell cultures	Pro-survival	ATG7 siRNA	[82]
mTHPC	ER	*In vitro* cell cultures	Pro-death	Wortmannin	[87]
Pc4	Mitochondria ER	*In vitro* cell cultures	Pro-death	ATG7/LC3 siRNA	[88]
Pc4	Mitochondria ER	*In vitro* cell cultures	Pro-death	3-MA Wortmannin	[89]
Pc4	Mitochondria ER	*In vitro* cell cultures	Pro-survival	ATG7 siRNA	[90]
Photofrin	Mitochondria Cellular Membranes	*In vitro* cell cultures	No effect	3-MA Bafilomycin A1	[91]
Platonin	?	*In vitro* cell cultures	Pro-death	3-MA	[92]
Protoporphyrin IX	Mitochondria	*In vitro* cell culture and *in vivo* implantation analysis	Pro-survival	CQ 3-MA ATG5 shRNA ATG3 shRNA	[54]
Rose Bengal Acetate	Cytoskeleton Mitochondria Golgi apparatus ER	*In vitro* cell cultures	Pro-death	3-MA	[80, 93]
Verteporfin	Mitochondria ER	*In vitro* cell cultures	Pro-survival	CQ ATG7 siRNA	[52, 53]
Chaperone-mediated Autophagy (CMA) and cancer's response to PDT					
Hypericin	ER	*In vitro* cell cultures	Pro-survival and Suppresses	LAMP2A knock-out -cells	[48, 57]

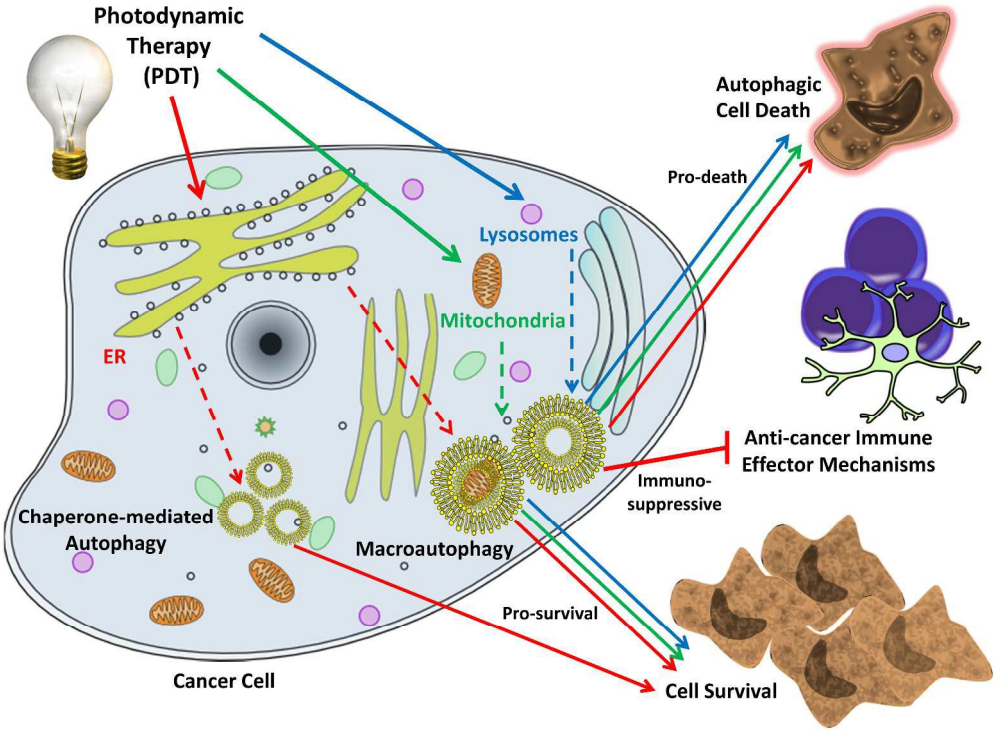
			'eat me' signal ecto-CRT		
3-MA – 3-methyladenine; 5-ALA - 5-aminolevulinic acid; CRT – calreticulin; Ecto – surface exposed; ER – endoplasmic reticulum; KO – knock-out; mTHPC – m-tetrahydroxyphenylchlorin; Pc 4 – (Silicon) Phthalocyanine;					

Figure Legend

Figure 1. The role of autophagy in cancer responsiveness or resistance to photodynamic therapy (PDT). Conditional to the photosensitiser under contemplation and the setting (but free of the photosensitiser's target subcellular organelle like the ER, mitochondria or lysosomes), autophagy triggered by PDT either adds to therapy resistance (pro-survival role confirmed for macroautophagy and chaperone-mediated autophagy) or vulnerability (pro-death role *via* autophagic cell death, largely through macroautophagy). Furthermore, cancer cell-associated autophagy also supports resistance against PDT by overpowering anticancer immune effector contrivances.

Figure 2. Pro-survival role of autophagy in cancer cells, following treatment with photodynamic therapy (PDT). Several photosensitisers, each with its specific subcellular localization, can induce pro-survival autophagy, after irradiation (see Table I and the main text for more detailed information). The light blue double membrane vesicle represents the autophagosome while the dark blue, designates the lysosome. Akt, mTOR and Bcl-2 are autophagy inhibitors. Bax, Cyt c (Cytocrome c) and Caspase 3 are involved in the apoptotic machinery. Abbreviations: Hyp, Hypericin; BPD, Benzo Porphyrin Derivative (Verteporfin); PC4, (silicon) PhthaloCyanine; CPO, 9-Capronyloxytetrakis (methoxy-ethyl)porphycene; MC, MesoChlorin; PpIX, Protoporphyrin IX; 5-ALA, 5-AminoLevulinic Acid; Ch f, Chlorofyllin f; Ch e4, Chlorofyllin e4.

Figure 3. Pro-death role of autophagy in cancer cells, following treatment with photodynamic therapy. Several photosensitisers, each with its specific subcellular localization, can induce autophagic cell death, after irradiation (see Table I and the main text for more detailed information). The light blue double membrane vesicle represents the autophagosome, while the dark blue designates the lysosome. In yellow is indicated an intracytoplasmic vesicle. Abbreviations: RBAc, Rose Bengal Acetate; PC4, (silicon) PhthaloCyanine; mTHPC, metaTetra-Hydroxy Phenyl Chlorin; 5-ALA, 5-AminoLevulinic Acid; HP, HematoPorphyrin; WST11, a water-soluble palladium bacteriopheophorbide; NPe6, N-aspartyl chlorin e6; GQD, Graphene Quantum Dots.



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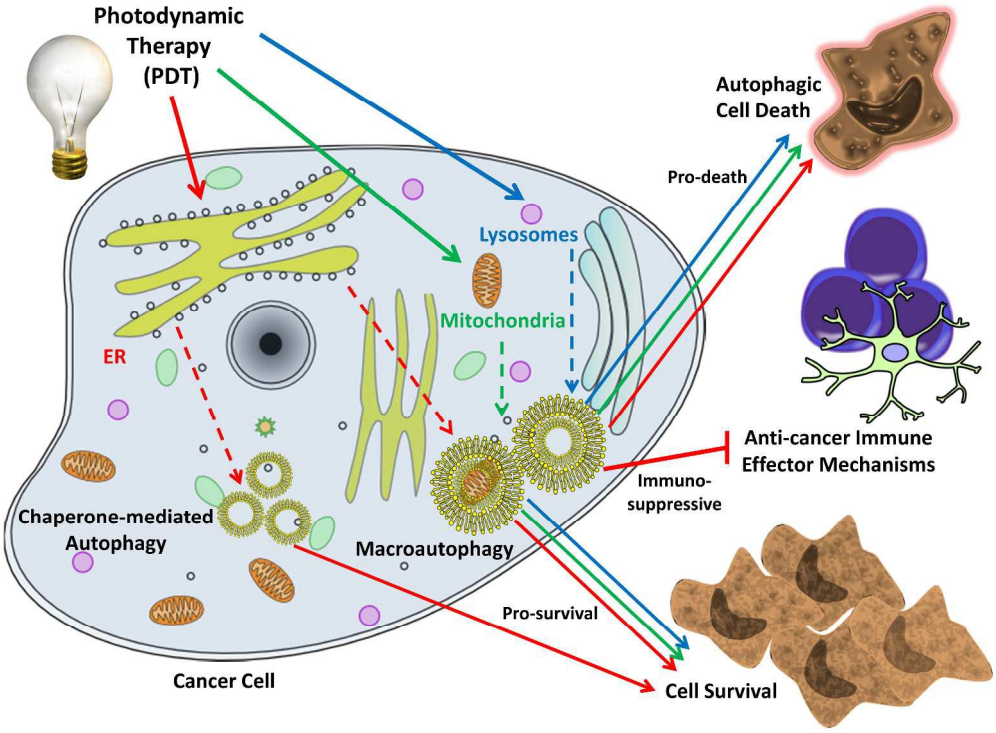

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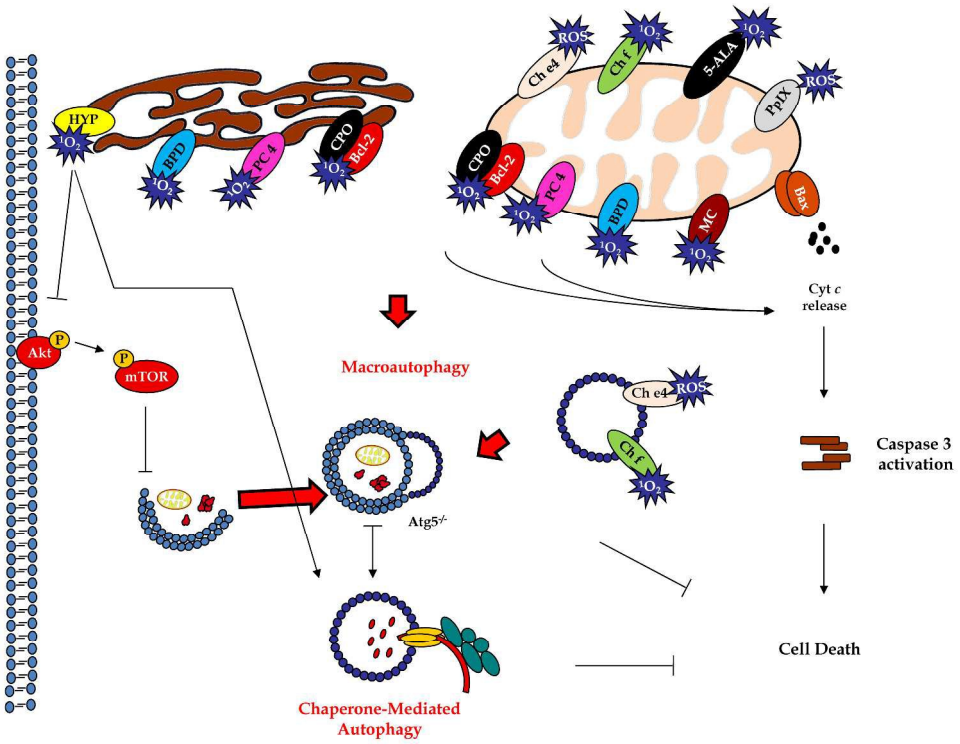

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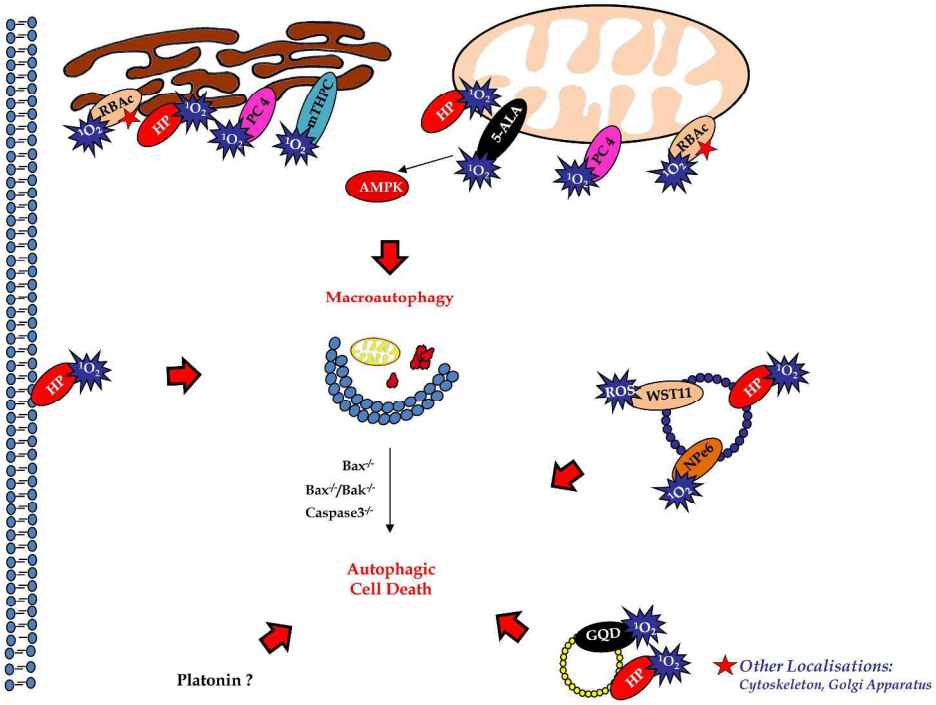

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